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(54) Title: MOLECULAR TYPING OF GROUP B STREPTOCOCCI

(57) Abstract: Molecular methods are provided for typing group B streptococci, as well as polynucleotides useful in such methods.

MOLECULAR TYPING OF GROUP B STREPTOCOCCI

Field of the invention

The present invention relates to molecular methods of typing group B streptococci, as well as polynucleotides useful in such methods.

Background to the invention

Group B streptococcus (GBS) - *Streptococcus agalactiae* - is the commonest cause of neonatal and obstetric sepsis and an increasingly important cause of septicaemia in the elderly and immunocompromised patients. The incidence of neonatal GBS sepsis has been reduced in recent years by the use of intrapartum antibiotic prophylaxis, but there are many problems with this approach. In future, vaccination is likely to be preferred and there has been considerable progress in development of conjugate polysaccharide GBS vaccines.

Before the introduction of conjugate vaccines, extensive epidemiological and other related studies will be required to assess, not only the burden of disease, but also the distribution of GBS types (including capsular polysaccharide gene serotypes, serosubtypes; protein antigen gene subtypes; mobile genetic element subtypes) to determine the optimal formulation of vaccine antigens. Type distribution based on one geographic location or small numbers of patients may not be generally applicable. Continued monitoring will be necessary to assess the suitability of combinations of GBS vaccine antigens for different target populations in different geographic locations.

Nine capsular polysaccharide GBS serotypes have been described (Harrison et al., 1998; Hickman et al., 1999). Various serotyping methods have been used, including immuno-precipitation (Wilkinson and Moody, 1969), enzyme immunoassay (Holm and Hakansson, 1988), coagglutination (Hakansson et al., 1992), counter-immunoelectrophoresis, and capillary precipitation (Triscott and Davies, 1979), latex agglutination (Zuerlein et al., 1991), fluorescence microscopy (Cropp et al., 1974) and inhibition-ELISA (Arakere et al., 1999). These methods are labour-intensive and require high-titered serotype-specific antisera, which are expensive and difficult to make and commercially available for only six serotypes - Ia to V (Arakere et al., 1999). Molecular genotyping methods, such as pulsed-field gel electrophoresis (Rolland et al., 1999), restriction endonuclease analysis (Nagano et al., 1991) are useful for epidemiological studies but do not generally identify serotypes. Consequently, there is a need for a reliable molecular method for GBS serotype identification.

Summary of the invention

We have identified specific regions within the genome of group B streptococci of inter-type sequence heterogeneity that can be used to distinguish different types (including capsular polysaccharide gene serotypes and serosubtypes; protein antigen gene subtypes; and mobile genetic element subtypes). We have shown that molecular methods that detect these sequence heterogeneities can be used to accurately distinguish and type group B streptococci.

Accordingly in a first aspect the present invention provides a method of typing a group B streptococcal bacterium which method comprises analysing the nucleotide sequence of one or more regions within the *cpsD, cpsE, cpsF, cpsG, cpsI/M* genes of said bacterium, said region(s) comprising one or more nucleotides whose sequence varies between types.

In particular, the nucleotide sequence may be analysed for one or more positions corresponding to positions 62, 78-86, 138, 139, 144, 198, 204, 211, 281, 240, 249, 300, 321, 419, 429, 437, 457, 466, 486, 602, 606, 627, 636, 645, 803, 971, 1026, 1044, 1173, 1194, 1251, 1278, 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

Preferably at least one region is within a sequence delineated by the 3' 136 bases of the *cpsE* gene and the 5' 218 bases of the *cpsG* gene of the *cpsE-cpsF-cspG* gene cluster of said group B streptococcal bacterium. In particular, the nucleotide sequence may be analysed for one or more positions corresponding to positions 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

In one embodiment, at least one region is within the cpsI/M genes of said group B streptococcal bacterium.

We have also shown that a number of surface protein antigen genes, including *rib, alp2* or *alp3* genes, and five mobile genetic elements may be used to molecular subtype GBS. Accordingly, the present invention also provides a method of typing a group B streptococcal bacterium which method comprises determining the presence or absence in the genome of said bacterium of one or more surface protein antigen genes selected from a *rib, alp2* or *alp3* gene, and/or one or more mobile genetic elements selected from IS*861*, IS*1548*, IS*1381*,

ISSa4 and GBSi1. Preferably, such as method is combined with the above methods of the invention.

The nucleotide sequence analysis step may comprise sequencing said one or more regions. Alternatively, or in addition, the nucleotide sequence analysis step may comprises determining whether a polynucleotide obtained from said bacterium selectively hybridises to a polynucleotide probe comprising one or more of the said regions, preferably to one or more of a plurality of polynucleotide probes corresponding to one or more of the said regions.

In a preferred embodiment, where hybridisation to a plurality of probes is used as a means of analysis, the plurality of polynucleotide probes are present as a microarray.

In another embodiment, the nucleotide sequence analysis step comprises an amplification step using one or more primers, at least one of which hybridise specifically to a sequence which differs between types. Typically, primer pairs are used, at least one of which hybridise specifically to a sequence which differs between types. Preferably, said primers are selected from the primers shown in Table 2 and/or Table 6 and/or Table 10.

In a second aspect, the present invention provides a polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within a *cpsD-cpsE-cpsF-cpsG* gene of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between GBS types.

Preferably the nucleotides which differ between GBS types correspond to one or more of positions 62, 78-86, 138, 139, 144, 198, 204, 211, 281, 240, 249, 300, 321, 419, 429, 437, 457, 466, 486, 602, 606, 627, 636, 645, 803, 971, 1026, 1044, 1173, 1194, 1251, 1278, 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

The present invention also provides a polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within a sequence delineated by the 3' 136 base pairs of *cpsE* and the 5' 218 base pairs of *cpsG* of the *cpsE-cpsF-cspG* gene cluster of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between GBS types.

Preferably the nucleotides which differ between group B streptococcal types correspond to one or more of positions 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

The present invention also provides a polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within a *cpsI/M* gene of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between group B streptococcal types.

Preferably the polynucleotide is selected from the nucleotide sequences shown in Table 2.

The present invention further provides a polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within a *rib, alp2* or *alp3* gene of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between GBS protein antigen gene subtypes.

Preferably the polynucleotide is selected from the nucleotide sequences shown in Table 6.

The present invention further provides a polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within IS*861*, IS*1548*, IS*1381*, ISSa*4* and/or GBSi1 of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between GBS mobile genetic element subtypes.

Preferably the polynucleotide is selected from the nucleotide sequences shown in Table 10.

The polynucleotides of the invention may be used in a method of typing, such as serotyping and/or subtyping, a group B streptococcal bacterium.

In a third aspect the present invention provides a composition comprising a plurality of polynucleotides of the second aspect of the invention. The composition may be used in a method of typing, such as serotyping and/or subtyping, a group B streptococcal bacterium.

In a fourth aspect the present invention provides a microarray comprising a plurality of polynucleotides according to the second aspect of the invention. The microarray may be used in a method of typing, such as serotyping and/or subtyping, a group B streptococcal bacterium.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular

Cloning: A Laboratory Manual, 3rd ed. (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

The molecular typing methods of the present invention rely on detecting the presence in sample of specific polynucleotide sequences in regions of the genome of group B streptococci (GBS) that we have identified as varying between different types.

More specifically, the specific polynucleotide sequences that are to be detected lie within *cpsD, cpsE, cpsF, cpsG, cpsI, cpsM, rib, alp2* and/or *alp3* genes of GBS as well as mobile genetic elements IS*861*, IS*1548* and IS*1381*, ISSa*4* and GBSi1, preferably the *cpsD, cpsE, cpsF, cpsG* and/or *cpsI/M* genes.

Regions of interest within those genes mentioned are regions whose sequence varies between two or more types, i.e. are heterogenous. Heterogeneity may be due to insertions, deletions and/or substitutions between corresponding regions in different types. In the case of *rib, alp2* and *alp3*, heterogeneity typically takes the form of the presence or absence of the entire gene. Similarly for elements IS*861*, IS*1548*, IS*1381*, ISSa*4* and GBSi1 heterogeneity typically takes the form of the presence or absence of the entire sequence.

Specific regions of heterogeneity include the following positions within *cpsD* gene- 62 and 78-86; *cpsD-cpsE* gene spacer - 138, 139 and 144; *cpsE* gene - 198, 204, 211, 281, 240, 249, 300, 321, 419, 429, 437, 457, 466, 486, 602, 606, 627, 636, 645, 803, 971, 1026, 1044, 1173, 1194, 1251, 1278, 1413, 1495, 1500, 1501, 1512, 1518 and 1527; *cpsF* gene - 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892 and 1971; and *cpsG* gene - 2026, 2088, 2134, 2187 and 2196 (numbering corresponds to numbering shown in Figure 1).

Particularly preferred positions of interest are those that lie within a 790 bp fragment of *cpsE-cps-F-cpsG* (which consists of approximately the 3' 136 bases of *cpsE* to the 5' 218 bases of *cpsG*), namely positions 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

Another region of heterogeneity is position 62 of *cpsD* and a repetitive sequence (TTACGGCGA) found at positions 78 to 86 of *cpsD* in some but not all GBS serotypes.

Specific regions of heterogeneity also include a number of positions within the *cpsI/M* gene as shown in the sequence alignment depicted in Figure 3.

These regions of heterogeneity may be analysed using a variety of means including sequencing, PCR and binding of labelled probes.

In the case of sequencing to identify serotype, the sequencing primers are selected such that they hybridise specifically to a region within or near to a region within which a region of heterogeneity is present. The primers need not be specific to particular serotypes since the actual sequence information obtained during the sequencing process which is used to assign molecular serotype. Thus the primers may hybridise specifically to all GBS serotypes (at least serotypes Ia to VII), or to specific serotypes.

Preferred primers anneal within 100, 50 or 20 contigous nucleotides of a heterogeneous position within the 790 bp region of *cpsE-cpsF-cpsG* shown in Figure 1. Examples of suitable sequencing primers are shown in Table 2 (cpsES3, cpsFA, cpsFS, cpsGA and cpsGA1).

PCR and other specific hybridisation- based serotyping methods will typically involve the use of nucleotide primers/probes which bind specifically to a region of the genome of a GBS serotype which includes a nucleotide which varies between two or more serotypes. Thus the primers/probes may comprise a sequence which is complementary to one of such regions. Where positions of heterogeneity are close together (e.g. positions 198, 204, 211 and 218 of *cpsE*), it may be desirable to use a primer/probe which hybridises specifically to a region of the GBS genome that comprises two or more positions of heterogeneity. Thus for example, a primer/probe may be designed that is complementary to nucleotides 195 to 220 of *cpsE*. Such primers/probes are likely to have improved specificity and reduce the likelihood of false positives.

PCR-based methods of detection may rely upon the use of primer pairs, at least one of which binds specifically to a region of interest in one or more, but not all, serotypes. Unless both primers bind, no PCR product will be obtained. Consequently, the presence or absence of a specific PCR product may be used to determine the presence of a sequence indicative of specific GBS serotypes. However, as mentioned, only one primer need correspond to a region of heterogeneity in the genes of interest (such as the *cpsD, cpsE, cpsF, cpsG, cpsI* and/or *cpsM* genes). The other primer may bind to a conserved or heterogenous region within said gene or even a region within another part of the GBS genome, such as the *cpsH* gene, whether said region is conserved or heterogeneous between serotypes. Thus, for example, a combination of a primer (cpsGS) which binds to a region of the *cpsG* gene including positions 2172 to 2210, and a primer

which binds to a region of *cpsH* gene which is heterogeneous (IacpsHA1, IIIcpsHA), may be used as the basis of distinguishing serotypes (Ia and III).

Further, a primer which binds to a region of *cpsI* which is heterogeneous may be combined with a primer which binds to a region of *cpsG* which is constant. An example of such as primer pair is primer pair VIcpsIA, and cpsGS1, which give rise to a PCR product of 1517 bp and GBS serotype VI specific.

Alternatively, primers that bind to conserved regions of the GBS genome but which flank a region whose length varies between serotypes may be used. In this case, a PCR product will always be obtained when GBS bacteria are present but the size of the PCR product varies between serotypes.

Furthermore, a combination of specific binding of one or both primers and variations in the length of PCR primer may be used as a means of identifying particular molecular serotypes.

Examples of specific primers/probes which target the *cpsD, cpsE, cpsF, cpsG, cpsI* or *cpsM* genes include the following:

cpsDS	GCA AAA GAA CAG ATG GAA CAA AGT GG
cpsES	CTT TTG GAG TCG TGG CTA TCT TG
cpsEA1	GA/T/GA AAA AAG GAA AGT CGT GTC G/ATT G
cpsES1	CTT GGA C/TTC CTC TGA AAA GGA TTG
cpsEA2	AAA A/CGC TTG ATC AAC AGT TAA GCA GG
cpsES2	GAT GGT/C GGA CCG GCT ATC TTT TCT C
cpsEA3	CTT AAT TTG TTC TGC ATC TAC TCG C
cpsES3	GTT AGA TGT TCA ATA TAT CAA TGA ATG GTC TAT TTG GTC AG
cpsEFA	CCT TTC AAA CCT TAC CTT TAC TTA GC
cpsFS	CAT CTG GTG CCG CTG TAG CAG TAC CAT T
cpsFA	GTC GAA AAC CTC TAT A/GT A AAC/T GGT CTT ACA A/GCC AAA TAA CTT ACC
cpsGA	AAG/C AGT TCA TAT CAT CAT ATG AGA G
cpsGA1	CCG CCA/G TGT GTG ATA ACA ATC TCA GCT TC
cpsGS	ATG ATG ATA TGA ACT CTT ACA TGA AAG AAG CTG AGA TTG
cpsGS1	GAA CTC TTA CAT GAA AGA AGC TGA GAT TGT TAT CAC AC
IbcpsIA	CTA TCA ATG AAT GAG TCT GTT GTA GGA CGG ATT GCA CG
IbcpsIS	GAT AAT AGT GGA GAA ATT TGT GAT AAT TTA TCT CAA AAA GAC G
IbcpsIA1	CCT GAT TCA TTG CAG AAG TCT TTA CGA TGC GAT AGG TG
IVcpsMA	GGG TCA ATT GTA TCG TCG CTG TCA ACA AAA CCA ATC AAA TC
VcpsMA	CCC CCC ATA AGT ATA AAT AAT ATC CAA TCT TGC ATA GTC AG

VIcpsIA GAA GCA AAG ATT CTA CAC AGT TCT CAA TCA CTA ACT CCG
cpsIA GTA TAA CTT CTA TCA ATG GAT GAG TCT GTT GTA GTA CGG

The primer designations correspond to those given in Table 2.

In relation to the *alp2, alp3* and *rib* surface protein antigen genes, heterogeneity and protein antigen gene subtype is assessed more at the level of whether a group B streptococcal bacterium contains the gene or not. Our results show that the specific combination of surface proteins genes present in a GBS genome is indicative of serotype/serosubtypes (see Table 9). Consequently, primers/probes suitable for use in the methods of the present invention are those that are specific for the particular genes. Thus probes/primers that are specific for *alp2* or *alp3* or *rib* are preferred. Figure 4 shows an alignment of *alp2* and *alp3* that was used to design primers specific for *alp2* or specific for *alp3*.

Examples of specific primers/probes which target the *alp2, alp3* and *rib* genes include the following:

bcaS1 GGT AAT CTT AAT ATT TTT GAA GAG TCA ATA GTT GCT GCA TCT AC
bcaS2 CCAGGGA GTG CAG CGA CCT TAA ATA CAA GCA TC
balS GAT CCT CAA AAC CTC ATT GTA TTA AAT CCA TCA AGC TAT TC
balA CCA GTT AAG ACT TCA TCA CGA CTC CCA TCA C
bal23S1 CAG ACT GTT AAA GTG GAT GAA GAT ATT ACC TTT ACG G
bal23S2 CTT AAA GCT AAG TAT GAA AAT GAT ATC ATT GGA GCT CGT G
bal2S CTT CCG CCA GAT AAA ATT AAG
bal2A CTG TTG ACT TAT CTG GAT AGG TC
bal2A1 CGT GTT GTT CAA CAG TCC TAT GCT TAG CCT CTG GTG
bal2A2 GGT ATC TGG TTT ATG ACC ATT TTT CCA GTT ATA CG
bal3S GTT CTT CCG CTT AAG GAT AG
bal3A GAC CGT TTG GTC CTT ACC TTT TGG TTC GTT GCT ATC C
ribS2 GAAGTAATTTCAG GAA GTG CTG TTA CGT TAA ACA CAA ATA TG
ribA1 GAA GGT TGT GTG AAA TAA TTG CCG CCT TGC CTA ATG
ribA2 AAT ACT AGC TGC ACC AAC AGT AGT CAA TTC AGA AGG

The primer designations correspond to those given in Table 6.

In relation to the IS*861*, IS*1548*, IS*1381*, ISSa*4* and GBSi1, heterogeneity and subtype is assessed more at the level of whether a group B streptococcal bacterium contains the element or not. The number of elements may also be assessed. Our results show that the specific combination of mobile elements present in a GBS genome is indicative of serotype/serosubtype (see Table 12).

Consequently, primers/probes suitable for use in the methods of the present invention are those that are specific for the particular mobile genetic elements. Thus probes/primers that are specific for IS*861*, IS*1548*, IS*1381*, ISSa*4* and GBSi1 are preferred.

Examples of specific primers/probes which target IS*861*, IS*1548*, IS*1381*, ISSa*4* and GBSi1 include the following:

IS861S	GAG AAA ACA AGA GGG AGA CCG AGT AAA ATG GGA CG
IS861A1	CAC GAT TTC GCA GTT CTA AAT AAA TCC GAC GAT AGC C
IS861A2	CAA ACT CCG TCA CAT CGG TAT AGC ACT TCT CAT AGG
IS1548S	CTA TTG ATG ATT GCG CAG TTG AAT TGG ATA GTC GTC
IS1548S1	GTT TGG GAC AGG TAG CGG TTG AGG AGA AAA GTA ATG
IS1548A1	CAT TAC TTT TCT CCT CAA CCG CTA CCT GTC CCA AAC
IS1548A2	CCC AAT ACC ACG TAA CTT ATG CCA TTT G
IS1548A3	CGT GTT ACG AGT CAT CCC AAT ACC ACG TAA CTT ATG CC
IS1381S1	CTT ATG AAC AAA TTG CGG CTG ATT TTG GCA TTC ACG
IS1381S2	GGC TCA GGC GAT TGT CAC AAG CCA AGG GAG
IS1381A	CTA AAA TCC TAG TTC ACG GTT GAT CAT TCC AGC
ISSa4S	CGT ATC TGT CAC TTA TTT CCC TGC GGG TGT CTC C
ISSa4A1	GCC GAT GTC ACA ACA TAG TTC AGG ATA TAG CCA G
ISSa4A2	CGT AAA GGA GTC CAA AGA TGA TAG CCT TTT TGA ACC
GBSi1S1	CAT CTC GGA ACA ATA TGC TCG AAG CTT ACA AGC AAG TG
GBSi1S2	GGG GTC ACT ATC GAG CAG ATG GAT GAC TAT CTT CAC
GBSi1A1	AAT GGC TGT TTC GCA GGA GCG ATT GGG TCT GAA CC
GBSi1A2	CCA GGG ACA TCA ATC TGT CTT GCG GAA CAG TAT CG

Preferably, the primers/probes comprise at least 10, 15 or 20 nucleotides. Typically, primers/probes consist of fewer than 100, 50 or 30 nucleotides. Primers/probes are generally polynucleotides comprising deoxynucleotides. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Primers/probes may be labelled with any suitable detectable label such as radioactive atoms, fluorescent molecules or biotin.

In one embodiment, primers/probes have a high melting temperature of >70°C so that they may be used in rapid cycle PCR.

Compositions comprising a plurality of nucleotides that are used to analyse one or more regions within the *cpsD, cpsE, cpsF, cpsG*, or *cpsI/M* genes may also further comprise nucleotides that may be used to analyse one or more regions within the *cpsH* gene. Suitable nucleotides are described in the Examples, although a person skilled in the art could design other suitable sequences based on the sequence alignment shown in Figure 3.

Further, compositions comprising a plurality of nucleotides that are used to analyse one or more regions within *alp2, alp3* or *rib* genes may also further comprise nucleotides that may be used to analyse one or more regions within the C alpha (*bca*) and C beta (*bac*) genes (C beta gene also known as *bag).*

A variety of techniques may be used to analyse one or more regions within the genome of a bacterium of interest. Typically, a sample of interest, which is suspected of containing GBS bacteria is treated, using standard techniques to obtain genomic DNA from any microorganisms present in the sample. It may be desirable for a number of subsequent detection steps to use nucleic acid preparation techniques that result in substantial fragmentation of the genomic DNA. The sample may be from a bacterial culture or a clinical sample from a patient, typically a human patient. Clinical samples may be cultured to produce a bacterial culture. However, it is also possible to test clinical samples directly with a culturing step.

The genomic DNA is then subjected to one or more analysis steps which may include sequencing, enzymatic amplification and/or hybridisation. These general techniques of DNA analysis are known in the art and are discussed in detail in, for example, Sambrook et al. 2001 and Ausubel et al. 1999 *supra.*

Serotyping may involve a one or more steps. For example, it may be desirable to carry out an initial step of determining whether there are nucleotide sequences present in the sample which are conserved between GBS seroptypes but not found in any other organism. This may be achieved by using PCR primers that detect any (but only) GBS bacteria (e.g. using primer pairs Sag59/Sag190 and/or DSF2/DSR1 - see Tables 2 and 3).

Molecular serotyping for specific GBS serotypes can then be performed by detecting the presence of one or more regions of heterogeneity in the regions of interest using any suitable technique such as sequencing, enzymatic amplification and/or hybridisation based on the probes/primers discussed above.

A particularly preferred detection technique is PCR, such as rapid cycle PCR (Kong et al., 2000).

An example of a multi-step serotyping strategy (algorithm) is shown in Figure 2. However, a variety of other strategies are envisaged and can be designed by the skilled person using the sequence heterogeneity information presented herein. In particular, it is preferred that the serotyping procedure comprise at least one analysis step based on analysing one or regions of the *cpsD, cpsE, cpsF, cpsG* and/or *cpsI/M* genes. This analysis may optionally be combined with an analysis of one or more regions within the *cpsH* gene. Similar techniques may be used to analyse the *cpsH* gene regions and suitable primer sequences and methods are also described in the Examples.

Analysis of the presence of absence of the *alp2, alp3* and/or *rib* genes may optionally be combined with an analysis of the presence or absence of C alpha (*bca* gene), C beta (*bac*) gene sequences as is described in the Examples. Similar techniques may be used to analyse these regions and suitable primer sequences and PCR methods are also described in the Examples.

Furthermore, analysis of the presence of absence of the *alp2, alp3* and/or *rib* genes (and optionally the *bca* and *bac* genes) may be combined with an analysis of the presence or absence of mobile genetic elements.

Thus a typing strategy may involve an analysis of *cps* genes, surface protein genes and/or mobile genetic elements in various combinations to provide more serosubtyping and subtyping information.

Analysis of GBS genomic sequences using the above techniques may take place in solution followed by standard resolution using methods such as gel electrophoresis. However in a preferred aspect of the invention, the primers/probes are immobilised onto a solid substrate to form arrays.

The polynucleotide probes are typically immobilised onto or in discrete regions of a solid substrate. The substrate may be porous to allow immobilisation within the substrate or substantially non-porous, in which case the probes are typically immobilised on the surface of the substrate. Examples of suitable solid substrates include flat glass (such as borosilicate glass), silicon wafers, mica, ceramics and organic polymers such as plastics, including polystyrene and polymethacrylate. It may also be possible to use semi-permeable membranes such as nitrocellulose or nylon membranes, which are widely available. The semi-permeable membranes may be mounted on a more robust solid surface such as glass. The surfaces may optionally be coated with a layer of metal, such as gold, platinum or other transition metal.

Preferably, the solid substrate is generally a material having a rigid or semi-rigid surface. In preferred embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be

desirable to physically separate synthesis regions for different polymers with, for example, raised regions or etched trenches. It is also preferred that the solid substrate is suitable for the high density application of DNA sequences in discrete areas of typically from 50 to 100 μm, giving a density of 10000 to 40000 cm^{-2}.

The solid substrate is conveniently divided up into sections. This may be achieved by techniques such as photoetching, or by the application of hydrophobic inks, for example teflon-based inks (Cel-line, USA). Discrete positions, in which each different probes are located may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc.

Attachment of the library sequences to the substrate may be by covalent or non-covalent means. The library sequences may be attached to the substrate via a layer of molecules to which the library sequences bind. For example, the probes may be labelled with biotin and the substrate coated with avidin and/or streptavidin. A convenient feature of using biotinylated probes is that the efficiency of coupling to the solid substrate can be determined easily. Since the polynucleotide probes may bind only poorly to some solid substrates, it is often necessary to provide a chemical interface between the solid substrate (such as in the case of glass) and the probes. Thus, the surface of the substrate may be prepared by, for example, coating with a chemical that increases or decreases the hydrophobicity or coating with a chemical that allows covalent linkage of the polynucleotide probes. Some chemical coatings may both alter the hydrophobicity and allow covalent linkage. Hydrophobicity on a solid substrate may readily be increased by silane treatment or other treatments known in the art. Examples of suitable chemical coatings include polylysine and poly(ethyleneimine). Further details of methods for the attachment of are provided in US Patent No. 6,248,521. Methods for immobilizing nucleic acids by introduction of various functional groups to the molecules are also described in Bischoff *et al.*, 1987 (Anal. Biochem., 164:336-3440 and Kremsky *et al.*, 1987 (Nucl. Acids Res. 15:2891-2910).

Techniques for producing immobilised arrays of nucleic acid molecules have been described in the art. A useful review is provided in Schena *et al.*, 1998, TibTech 16: 301-306, which also gives references for the techniques described therein.

Microarray-manufacturing technologies fall into two main categories—synthesis and delivery. In the synthesis approaches, microarrays are prepared in a stepwise fashion by the *in situ* synthesis of nucleic acids from biochemical building blocks. With each round of synthesis, nucleotides are added to growing chains until the desired length is achieved. A number of prior art methods describe

how to synthesise single-stranded nucleic acid molecule libraries *in situ*, using for example masking techniques (photolithography) to build up various permutations of sequences at the various discrete positions on the solid substrate. U.S. Patent No. 5,837,832 describes an improved method for producing DNA arrays immobilised to silicon substrates based on very large scale integration technology. In particular, U.S. Patent No. 5,837,832 describes a strategy called "tiling" to synthesize specific sets of probes at spatially-defined locations on a substrate which may be used to produced the immobilised DNA libraries of the present invention. U.S. Patent No. 5,837,832 also provides references for earlier techniques that may also be used.

The delivery technologies, by contrast, use the exogenous deposition of preprepared biochemical substances for chip fabrication. For example, DNA may also be printed directly onto the substrate using for example robotic devices equipped with either pins (mechanical microspotting) or piezo electric devices (ink jetting). In mechanical microspotting, a biochemical sample is loaded into a spotting pin by capillary action, and a small volume is transferred to a solid surface by physical contact between the pin and the solid substrate. After the first spotting cycle, the pin is washed and a second sample is loaded and deposited to an adjacent address. Robotic control systems and multiplexed printheads allow automated microarray fabrication. Ink jetting involves loading a biochemical sample, such as a polynucleotide into a miniature nozzle equipped with a piezoelectric fitting and an electrical current is used to expel a precise amount of liquid from the jet onto the substrate. After the first jetting step, the jet is washed and a second sample is loaded and deposited to an adjacent address. A repeated series of cycles with multiple jets enables rapid microarray production.

In one embodiment, the microarray is a high density array, comprising greater than about 50, preferably greater than about 100 or 200 different nucleic acid probes. Such high density probes comprise a probe density of greater than about 50, preferably greater than about 500, more preferably greater than about 1,000, most preferably greater than about 2,000 different nucleic acid probes per cm^2. The array may further comprise mismatch control probes and/or reference probes (such as positive controls).

Microarrays of the invention will typically comprise a plurality of primers/probes as described above. The primers/probes may be grouped on the array in any order. However, it may be desirable to group primers/probes according to types (capsular polysaccharide gene serotypes, serosubtypes; protein antigen gene subtypes; mobile genelic elements subtypes), or groups of types (capsular polysaccharide gene serotypes, serosubtypes; protein antigen gene subtypes; mobile genelic elements subtypes) for which they are specific.

Such grouping may be arranged such that the resulting patterns are easily susceptible to pattern recognition by computer software.

Elements in an array may contain only one type of probe/primer or a number of different probes/primers.

Detection of binding of GBS genomic DNA to immobilised probes/primers may be performed using a number of techniques. For example, the immobilised probes which are specific to a number of types (capsular polysaccharide gene serotypes, serosubtypes; protein antigen gene subtypes; mobile genelic elements subtypes), may function as capture probes. Following binding of the genomic DNA to the array, the array is washed and incubated with one or more labelled detection probes which hybridise specifically to regions of the GBS genome which are conserved. The binding of these detection probes may then be determined by detecting the presence of the label. For example, the label may be a fluorescent label and the array may be placed in an X-Y reader under a charge-coupled device (CCD) camera.

Other techniques include labelling the genomic DNA prior to contact with the array (using nick-translation and labelled dNTPs for example). Binding of the genomic DNA can then be detected directly.

It is also possible to employ a single PCR amplification step using labelled dNTPs. In this embodiment, the genomic DNA fragment binds to a first primer present in the array. The addition of polymerase, dNTPs, including some labelled dNTPs and a second primer results in synthesis of a PCR product incorporating labelled nucleotides. The labelled PCR fragment captured on the plate may then be detected.

A number of available detection techniques do not require labels but instead rely on changes in mass upon ligand binding (e.g. surface plasmon resonance- SPR). The principles of SPR and the types of solid substrates required for use in SPR (e.g. BIACore chips) are described in Ausubel *et al.*, 1999, supra.

C. Uses

As discussed above, group B streptococcus (GBS) - *Streptococcus agalactiae* - is the commonest cause of neonatal and obstetric sepsis and an increasingly important cause of septicaemia in the elderly and immunocompromised patients. Thus, the detection methods, probes/primer and microarrays of the invention may be used in the diagnosis of GBS infections in pregnant women, elderly and/or immunocompromised patients. The PCR and

microarray techniques described herein may be of particular use in routine antenatal screening of pregnant women as well as in diagnosing infections in pregnant women given the increased accuracy and sensitivity compared to conventional identification and serotyping. These methods are also likely to give faster results since it will not generally be necessary to culture clinical samples to obtain enough material. Further, the molecular techniques can be used in most laboratories without the need for specialist expertise or reagents.

The molecular typing methods of the invention may also assist in comprehensive strain identification that will be useful for epidemiological and other related studies that will be needed to monitor GBS isolates before and after introduction of GBS conjugate vaccines.

The present invention will now be described in more detail with reference to the following examples, which are illustrative only and non-limiting. The examples refer to Figures:

Detailed description of the Figures.

Figure 1. Molecular serotype identification based on the sequence heterogeneity of the 3'-end of *cpsD-cpsE-cpsF*-and the 5'-end of cps*G* (relevant primers are shown).

Figure 2. Algorithm for GBS molecular serotype (MS) identification by PCR and sequencing.

Figure 3. Multiple sequence alignments of the gene sequences of *cpsG-cpsH-cpsI/M* for serotypes Ia, Ib, II, III, IV, V and VI (start and stop codons are highlighted in bold).

Figure 4. Two sites (*) of sequence heterogeneity between *alp2* (AF208158, upper lines) and *alp3* (AF291065, lower lines) used to distinguish them (relevant primers are shown).

Figure 5. Genetic relationship of 194 invasive Australasia GBS strains (or 56 genotypes).

Notes for column headed "Genetic Markers of GBS genotypes":
Protein antigen gene profile codes are:

"A": 5'end of *bca* positive;
"a" or "as": *bca* repetitive unit or *bca* repetitive unit-like region positive, with multiple or single band amplicons, respectively;
"B": *bac* positive;
"R": *rib* positive;
"alp2": *alp2* positive;
"alp3": *alp3* positive;
"None": isolate contains none of the above protein genes.
The molecular markers in bold type show the common features in each cluster.

Notes for column headed "No. of strains":
After "+" are the numbers of CSF isolates, the others are blood isolates.

Notes for column headed "Genotypes":
Each genotype was characterized by a distinct combination of the *cps* genes, protein gene profiles and mobile genetic elements. The predominant genotype in each serotype were named as the number "1" genotype of that serotype.

Notes for the dendrogram:
At about distance 16, the 56 genotypes could be separated into 8 clusters (1-8); at about distance 22.5 the 56 genotypes could be separated into 3 cluster groups (A, B, C).

EXAMPLES

MATERIALS AND METHODS

GBS reference strains and clinical isolates.

A panel of nine GBS serotypes (Ia to VIII) was kindly provided by Dr Lawrence Paoletti, Channing Laboratory, Boston USA (reference panel 1). Dr Diana Martin, Streptococcus Reference Laboratory, at ESR, Wellington, New Zealand, provided another panel of nine international reference GBS type-strains including serotypes Ia to VI (reference panel 2) (Table 1). In addition, we tested isolates from 205 clinical cases including 146 which had been referred from various laboratories in New Zealand for serotyping and 59 isolated from normally sterile sites over a period of 10 years in one diagnostic laboratory in Sydney. One culture was subsequently shown to be mixed, so 206 different isolates were examined. Conventional serotyping (CS) was performed at the Streptococcus

Reference Laboratory, at ESR, Wellington, New Zealand, and MS at the Centre for Infectious Diseases and Microbiology Laboratory Services, ICPMR, Sydney, Australia.

The two panels of GBS reference strains and 63 selected clinical isolates were studied in more detail, by sequencing >2200 base pairs (bp) of each to identify appropriate sequences for use in MS. These and the remaining clinical isolates were then used to evaluate the MS method and compare results with those of CS. Typing by both methods was done initially without knowledge of results of the other.

Bacterial isolates were retrieved from storage by subculture on blood agar plates (Columbia II agar base supplemented with 5% horse blood) and incubated overnight at 37°C.

Invasive GBS clinical isolates

All 194 isolates used in the study of mobile genetic elements were recovered from the blood (177) or CSF (17) of 191 patients (107 female, 80 male, four sex unrecorded; three cultures each contained mixed growth of two GBS serotypes). 108 isolates were from specimens submitted for culture to the Centre for Infectious Diseases and Microbiology Laboratory Services, ICPMR, Sydney, Australia during 1996-2001 and 83 were referred to Institute of Environmental Science and Research (ESR), Porirua, Wellington, New Zealand for serotyping, from various diagnostic laboratories in New Zealand, during 1994-2000.

Patients were classified into age-groups for analysis of genotype distribution as follows: neonatal, early onset (0-6 days); neonatal, late onset (7 days to 3 months); infant and child (4 months-14 years); young adult (15-45 years); middle-aged (46-60 years); elderly (>60 years).

These isolates are mainly a subset of the isolates described above but with reference strains and non-invasive isolates excluded.

Conventional serotyping (CS).

CS was performed using standard methodology (Wilkinson and Moody, 1969). Briefly, an acid-heated (56°C) extract was prepared for each isolate and the serotype determined by immuno-precipitation of type-specific antiserum in agarose. An isolate was considered positive for a particular serotype when the precipitation occurring formed a line of identity with that of the control strain. Antisera used were prepared at ESR in rabbits against serotypes Ia, Ib, Ic, II, III, IV, V and the R protein antigen. Fourteen selected isolates, including six that

were nontypable using antisera against serotypes I-V, six that initially gave discrepant results between CS and MS and two separate isolates from a mixed culture, were kindly tested using antisera against all serotypes by Abbie Weisner and Dr. Androulla Efstratiou at Central Public Health Laboratory, Colindale, London, UK.

Molecular serotype identification (MS); development of method.

Oligonucleotide primers.

The oligonucleotide primers used in this study, their target sites and melting temperatures are shown in Tables 2, 6 and 10. Their specificities and expected lengths of amplicons are shown in Tables 3, 7 and 11. The primers were synthesised according to our specifications by Sigma-Aldrich (Castle Hill NSW, Australia). Four previously published oligonucleotide primers, and a series of new primers designed by us were used to sequence the genes of interest, namely 16S/23S rRNA intergenic spacer region and partial cps gene cluster, or to amplify unique sequences of individual GBS serotypes. Six previously published oligonucleotide primers and a series of new primers designed by us were used to sequence parts of and/or to specifically amplify genes encoding GBS surface proteins. We also designed a series of primers to sequence parts of and/or to specifically amplify five known GBS mobile genetic elements. Some were designed with high melting temperatures (>70 °C) to be used in rapid cycle PCR.

DNA preparation and polymerase chain reaction (PCR).

Five individual GBS colonies or a sweep of culture were sampled using a disposable loop and resuspended in 1 ml of digestion buffer (10mM Tris-HCl, pH 8.0, 0.45% Triton X-100 and 0.45% Tween 20) in 2 ml Eppendorf tubes. The tubes containing GBS suspension were heated at 100°C (dry block heater or water bath) for 10 minutes then quenched on ice and centrifuged for 2 minutes at 14,000 rpm to pellet the cell debris. 5 µL of each supernatant containing extracted DNA was used as template for PCR (Mawn et al., 1993).

PCR systems (25µL for detection only, 50 µL for detection and sequencing) were used as previously described (Kong et al., 1999). The denaturation, annealing and elongation temperatures and times used were 96°C for 1 second, 55-72°C (according to the primer Tm values or as previously described) for 1 second and 74°C for 1 to 30 seconds (according to the length of amplicons), respectively, for 35 cycles.

10 µL of PCR products were analysed by electrophoresis on 1.5 % agarose gels, which were stained with 0.5 µg ethidium bromide mL^{-1}. For detection and/or serotype identification, the presence of PCR amplicons of expected length, shown by ultraviolet transillumination, were accepted as positive. For sequencing, 40 µL volumes of PCR products were further purified by polyethylene glycol precipitation method (Ahmet et al., 1999).

Sequencing.

The PCR products were sequenced using Applied Biosystems (ABI) *Taq* DyeDeoxy terminator cycle-sequencing kits according to standard protocols. The corresponding amplification primers or inner primers were used as the sequencing primers.

Multiple sequence alignments and sequence comparison.

Multiple sequence alignments were performed with Pileup and Pretty programs in Multiple Sequence Analysis program group. Sequences were compared using Bestfit program in Comparison program group. All programs are provided in WebANGIS, ANGIS (Australian National Genomic Information Service), 3rd version.

Surface protein gene profile codes

Each isolate was given a protein gene profile code according to positive PCR results using various primer pairs, as shown in Table 7.

Nucleotide sequence accession numbers.

The new sequence data described have been submitted to the GenBank Nucleotide Sequence Databases and allocated the following accession numbers: AF291411-AF291419 (16S/23S rRNA intergenic spacer regions for serotypes Ia to VIII reference strains from reference panel 1); AF332893-AF332917, AF363032-AF363060, AF367973, AF381030 and AF381031 (partial *cps* gene clusters for two panels of reference strains (Table) and selected representative clinical isolates); AF367974 (partial *bac* gene sequence, with an insertion sequence IS*1381* from one isolate), AF362685-AF362704 (partial *bac* gene sequences for all *bac*-positive isolates) and AF373214 (partial *rib*-like gene for reference strain Prague 25/60, an R protein standard strain).

Previously reported sequence data referred to herein have appeared in the GenBank Nucleotide Sequence Databases with the following accession numbers: AB023574 (16S rRNA gene); U39765, L31412 (16S/23S rRNA intergenic spacer

regions); X68427 (*S. oralis* 23S rRNA gene); X72754 (*cfb* gene); AB028896 (*cps* gene cluster for serotype Ia); AB050723 (partial *cps* gene cluster for serotype Ib); AF163833 (*cps* gene cluster for serotype III); AF355776 (*cps* gene cluster for serotype IV); AF349539 (*cps* gene cluster for serotype V); AF337958 (*cps* gene cluster for serotype VI); M97256 (*bca* gene); X58470, X59771 (*bac* gene); U58333 (*rib* gene); AF208158 (*alp2* gene), AF291065-AF291072 (*alp3* gene); AF064785 (IS*1381*); M22449 (IS*861*); Y14270 (IS*1548*); AF064785 (IS*1381*); AF165983 (ISSa*4*); and AJ292930 (GBSi1).

Statistical analysis and dendrogram.

SSPS version 11 software was used for statistic analysis. A dendrogram was formed using Average Linkage (between groups) and Hierarchical Cluster Analysis in SSPS version 11 software. The presence or absence of each marker - MS Ia, Ib, II, IV-VI , sst III-1-4; pgp "A", "R", "a", "as", "alp2", alp3"; *bac* subgroups 1, 1a, 2, 3, 3a, 3b, 3c, 4, 4b, 5a, 7, 7a, 8, 9, 9a, 10, n1, n2; and mge IS*1381*, IS*861*, IS*1548*, ISSa*4*, GBSil - were included in the analysis. The genotypes were each characterized by a distinct combination of the molecular serotyping (MS) or sst, pgp and mge.

Example 1 - Study of inter- and intra-serotype/serosubtype sequence heterogeneity in specific regions of the GBS genome and assessment of suitability for molecular serotyping/serosubtyping.

Polymerase chain reaction.

With two exceptions, all GBS-specific primer pairs produced amplicons of the expected size from all reference strains and clinical isolates tested (Table 3). The exceptions were Sag59/Sag190 and CFBS/CFBA. Both target the *cfb* gene, but failed to produce amplicons from one clinical isolate, despite repeated attempts. We assumed that this isolate either lacked the *cfb* gene or that the gene was present in a mutant form. It has been suggested previously that PCR targeting the *cfb* gene will not identify all GBS isolates (Hassan et al., 2000) and that another primer pair based on 16S rRNA gene, DSF2/DSR1 (Ahmet et al., 1999) was not entirely specific. Therefore, in this study, we used both primer pairs (DSF2/DSR1 and Sag59/Sag190) to confirm all the isolates were GBS.

Sequence heterogeneity of 16S/23S rRNA intergenic spacer regions.

The 16S/23S rRNA intergenic spacer regions were sequenced for the serotypes Ia to VIII from reference panel 1. Multiple sequence alignment showed

differences between serotypes at only two positions: 207 (serotype V is T or C [T/C], serotypes VII and VIII are C, others are T) and 272 (serotype III is T, others G). These regions are therefore unsuitable for MS.

Sequence heterogeneity at the 3'-end of *cpsD-cpsE-cpsF*-and the 5'-end of *cpsG*.

Using a series of primers targeting the 3'-end of *cpsD-cpsE-cpsF*-and the 5'-end of *cpsG*, we amplified and sequenced 2226 or 2217 bp - depending on the presence or absence of a nine-base repetitive sequence - from both panels of reference strains (serotypes Ia to VII) and 63 selected clinical isolates. Representative sequences were deposited into GenBank. See Table 1 for GenBank accession numbers of reference panel strains.

Repetitive sequence.

At the 3'-end region of *cpsD*, we found a nine-base repetitive sequence (TTA CGG CGA) in most isolates of MS Ia and II, some of MS III, all of MS IV, V, and VII, but none of the isolates of MS Ib or VI examined. (Table 4). The presence or absence of this repetitive sequence can be used to further subtype MS Ia, II and III (see below).

Intra-serotype heterogeneity.

In general, intra-serotype heterogeneity was low - there were minor random variations in a few isolates of all serotypes except MS III, in which the intra-serotype heterogeneity was more complex. MS III could be divided into four sequence subtypes on the basis of heterogeneity at 22 positions - 62, 139, 144, 204, 300, 321, 429, 437, 457, 486, 602, 636, 971, 1026, 1194, 1413, 1501, 1512,1518, 1527, 1629, and 2134 - and the presence or absence of the repetitive sequence (at 78-86) (Table 4).

Among 60 MS III isolates (58 clinical isolates and two reference strains), serosubtypes III-1 (30 isolates) and III-2 (22 isolates) were predominant. The repetitive sequence was present in serosubtype III-1 but not III-2; there were differences at seven other sites (139, 144, 204, 300, 321, 636, and 1629).

There were five isolates belonging to serosubtype III-3, which contained the repetitive sequence and were identical with serosubtype III-1 at three variable sites (139, 144, and 300) and with serosubtype III-2 at four (204,321, 626 and 1629). Seroubtype III-3 differed from both serosubtypes III-1 and III-2 at seven sites (486, 1026, 1413, 1512, 1518, 1527, and 2134). These seven sites in serosubtype III-3 were identical with the corresponding sites of MS Ia.

There were three serosubtype III-4 isolates, whose sequences were nearly identical with the corresponding sequence of MS II. The only exception was at position 437, where the nucleotide was T in serosubtype III-4 (as in MS VII), and C in MS II. This difference can be used (in addition to PCR, see below) to differentiate serosubtype III-4 from MS II. Two serosubtype III-4 isolates contained the repetitive sequence, and the other did not. Because of the small number of serosubtype III-4 isolates, we did not use the repetitive sequence to subtype them further.

Inter-serotype heterogeneity.

There were 56 sites of heterogeneity between the eight MS (Table 4). The most suitable sites, for use in PCR/sequencing for MS, were a group of 23 sites nearest to the 3'-end of the region (Table 4, Figure 1). Firstly, they were consistent across two panels of reference strains and most clinical isolates (the only exceptions were the small number of serosubtypes III-3 and III-4 isolates, see below). Secondly, they were relatively concentrated within a 790 bp region, which is a convenient length for sequencing in a single reaction. Thirdly, they contained enough heterogeneity sites to allow differentiation, with few exceptions, of MS Ia-VII. Based only on this 790 bp region, serosubtype III-3 cannot be distinguished from MS Ia, nor serosubtype III-4 from MS II. However, they can be identified by MS III-specific PCR (see below).

Serotype VIII does not form amplicons with primer pairs targeting the 790 bp region, but can be identified by exclusion after PCR identification of GBS. In this study, one MS VIII isolate was identified, for which none of the primer pairs that amplify the 2226 bp region (in addition to those that amplify the 790 bp region) produced amplicons. This result was confirmed by the use of serotype VIII-specific antiserum.

Mixed serotype-specificities in single isolates.

Eleven isolates were identified as one MS on the basis of the MS-specific PCR and overall sequence (within the 2226/2217 bp segment) but their sequences differed at some sites from isolates of the same MS and shared site-specific characteristics of another. They included five serosubtype III-3 isolates and three serosubtype III-4 (see above). One non-serotypable reference strain (Prague 25/60), which was identified as MS II, differed from other MS II isolates at five sites at the 5'-end of the region, and was identical with MS III at three of these sites. Prague 25/60 MS III-specific PCR was negative. One clinical isolate identified as CS II, and MS II on the basis of its overall sequence, had bases at

nine sites at the 5'-end of the region, that were characteristic of serotype Ib; MS Ib-specific PCR was negative. Finally, one CS V reference strain (Prague 10/84) had the same sequencing result as the corresponding sequence in GenBank (AF349539), but both were different, at three sites at the 5'-end of the region, from sequences of the other MS V strains that we studied.

All of these mixed-serotype specificities, except for those associated with serosubtypes III-3 and III-4, occurred at the 5'-end region of the 2226/2217 fragment. This supported our selection of the 790 bp 3'-end as the sequencing target for MS. Using this target, all MS were correctly identified except for MS III belonging to serosubtypes III-3 and III-4, which can be identified by MS III-specific PCR (see Example 2).

Example 2 - Molecular serotype identification (MS) based on MS-specific PCR targeting the 3'-end of *cpsG-cpsH-cps I/cpsM*.

Our sequence alignment results showed that there was significant sequence heterogeneity in the 3'-end of *cpsG-cpsH-cps I/cpsM* (Figure 3), which makes it appropriate for use in the design of specific primer pairs for differentiation of serotypes Ia, Ib, III, IV, V, and VI directly by PCR. To fulfil possible additional future requirements - for example, development of multiplex PCR and/or to allow further evaluation of the sequence typing method, we designed several primer pairs for each serotype (Tables 2 & 3). Using two panels of reference strains and the specified conditions, all primer pairs amplified DNA only from the corresponding serotypes. When clinical isolates were tested, similar results were obtained with two sets of MS-specific primer pairs. In general, more stringent conditions (lower primer concentration, higher annealing temperatures) could be used with primers generating smaller amplicons. Those selected for MS are shown in Table 3 and Figure 2.

A MS was assigned, by PCR, to 179 of 206 (86.9%) clinical isolates as follows: MS Ia 40; MS Ib 35; MS III 58 (including those previously identified as serosubtypes III-3 and III-4); MS IV 7; MS V 36; MS VI 3.

Example 3 - Comparison of serotype identification results between MS and CS.

After CS and MS had been completed, the results were compared. Initial results were discrepant for 15 isolates, all but five of which (see below) were resolved by retesting and/or correction of clerical errors.

The CS and MS/sequence subtyping results are shown in Table 5. A MS was assigned to all isolates by PCR and/or sequencing, compared with 188 of 206 (91.3%) by CS. Specific PCR has not yet been developed for MS II and VIII, so all MS II isolates were determined by sequencing only and one presumptive MS VIII isolate was decided by exclusion (see Example 1). For all other isolates, the results of PCR and sequencing were consistent, except for serosubtypes III-3 and III-4 and other minor sequence differences described above (Example 1). CS results correlated well with PCR results.

Final CS and MS results were the same for all 188 isolates (100%) for which results for both methods were available. Eighteen clinical isolates that were non-serotypable by CS, were assigned MS as follows: Ia, two; Ib, five; II, one; serosubtype III-1, three; serosubtype III-2, one; V, five; and VI, one.

Sequences (2217 bp) of three clinical isolates that we identified as MS VI, were identical with those for serotype VI reference strains and the corresponding sequence in GenBank (AF337958).

Mixed culture.

Four clinical isolates gave positive results with MS III-specific PCR, but were provisionally identified as MS II by sequencing. Three were CS III and one CS II, with a weak cross-reaction with serotype III antiserum. These isolates were studied further by subculturing 12 individual colonies of each. All subcultures were tested by MS III-specific PCR. All 12 colony subcultures of the three CS III isolates were positive by MS III-specific PCR and the isolates were therefore classified as serosubtype III-4 (see above). However, 11 of 12 colony subcultures of the fourth isolate were negative by MS III-specific PCR; and one was positive by MS III-specific PCR. It was therefore assumed that this was a mixed culture, predominantly of MS/CS II. The one MS III-specific PCR positive colony was subsequently identified as serosubtype III-2 and included as an additional clinical isolate (total 206 in all).

Example 4 - Algorithm for serotype assignment of GBS by PCR and sequencing

As an example of how the PCR and sequencing methods described above may be used clinically to perform GBS serotype identification, we designed an algorithm for clinical use. All the primers (except the inner sequencing primers) used were given high melting temperature (>70 °C), so rapid cycle PCR could be used (Figure 2) (see Table 2 for primer sequences).

Example 5 - Identification of regions in the *alp2, alp3* and *rib* genes suitable for protein antigen gene specific subtyping

Polymerase chain reactions.

With few exceptions, all primer pairs produced amplicons of predicted length from isolates giving positive results (Table 7). The exceptions included one isolate that was positive by PCR using primer pairs GBS1360S/GBS1937A and GBS1717S/GBS1937A (which both target *bac* gene) but produced amplicons significantly longer than those of other *bac* gene-positive isolates. Sequencing showed that the amplicon contained the insertion sequence IS*1381* with minor variations compared with the published sequences (Tamura et al., 2000). The amplicons produced using primers IgAagGBS/RIgAagGBS and IgAS1/IgAA1 (also targeting *bac* gene) varied in length (Berner et al., 1999) and were sequenced for further subtyping (see below and Table 8).

Amplicon sequencing results.

To confirm the specificity of selected primer pairs that we had designed or modified, we sequenced 10 of 23 amplicons produced by bcaS1/bcaA (targeting the 5'-end of *bca* gene) and all of those produced by ribS1/ribA3 (targeting *rib* gene) and GBS1360S/GBS1937A (targeting *bac* gene), from the two panels of reference strains and 31 randomly selected clinical isolates. .

All 10 amplicons of primers bcaS1/bcaA and 12 of 13 of primers ribS1/ribA3 were identical with the corresponding gene sequences in GenBank (M97256, *bca* gene and U58333, *rib* gene, respectively). One additional isolate, namely Prague 25/60 in reference panel 2 (which is used to raise R antiserum), produced an amplicon with primer pair ribS1/ribA3 only at a lower annealing temperature (55 °C) but not with ribS2/ribA1 and ribS2/ribA2. It was therefore assumed not to contain *rib* gene, although the amplicon sequence showed considerable homology with *rib* gene (71.4% or 66.6% according to whether or not the primer sequences were included) (Figure 3). This isolate was the only

one, of 224 tested, for which PCRs were negative using ribS2/ribA1 and ribS2/ribA2 but positive using ribS1/ribA3. The latter primer pair is assumed to be not entirely specific for *rib* gene and was therefore used only for sequencing.

Four of 10 amplicons of primer pair GBS1360S/GBS1937A (targeting *bac* gene) were identical with the corresponding sequence in GenBank (X58470, X59771). A single point mutation (A to G, 1441 of X59771) was found in the remaining six *bac* gene amplicons, including the one which contained the insertion sequence IS*1381* (see above and AF367974).

Amplicons from all of the 224 isolates that gave positive PCR results using primer pairs bcaS1/balA (targeting *alp2*/*alp3* genes), bal23S1/bal2A2 (targeting *alp2* gene) and IgAagGBS/RIgAagGBS (targeting *bac* gene) were sequenced.

Fifty isolates produced amplicons using primer pair bcaS1/balA. The sequences of nine were identical with the corresponding portions of the published sequence of *alp2* gene (AF208158) and 41 with that of *alp3* gene (AF291065). There are two consistent heterogeneity sites between *alp2* and *alp3* genes in the sequences of bcaS1/balA amplicons (Figure 4), which can be used to distinguish them, in addition to *alp2* and *alp3* gene -specific PCR. All nine amplicons of primer pair bal23S1/bal2A2 were identical with the corresponding portion of the *alp2* gene sequence in GenBank (AF208158).

The primer pair IgAagGBS/RIgAagGBS identified *bac* gene in 52 isolates. There was considerable sequence variation, which allowed separation of *bac* gene -positive isolates into 11 groups and 20 subgroups based on amplicon length and sequence heterogeneity, respectively (Table 8). The groups contained small numbers (one to five) of isolates except for B1 (20 isolates, 2 subgroups) and B4 (11 isolates, 3 subgroups). The differences in amplicon length was generally caused by the presence or absence of short repetitive sequences.

Further confirmation of specificity of surface protein gene-specific primer pairs.

To confirm primer specificity, we compared the results of PCR using the primer sequences we had designed or modified for *bac* gene PCR, with those of PCR using previously published primers and found 100% correlation.

The previously reported non-specificity of the published primer pair bcaRUS/bcaRUA (targeting the *bca* gene repetitive unit) was confirmed. Using these primers, all nine *alp2* gene positive (bcaS1/bcaA negative) isolates and 53 which were PCR negative using the primers bcaS1/bcaA, bcaS2/bcaA (targeting the 5'-end of *bca* gene), bal23S1/bal2A2 and bal23S2/bal2A1 (targeting the 5'-end of *alp2* gene) produced amplicons. Our sequencing showed that *bca* gene

and *alp2* gene have significant homology in the regions targeted by bcaRUS/ bcaRUA allowing amplicon formation from *alp2* gene -positive strains. These false positive results could be due to the presence of other C alpha-like proteins, containing regions homologous with the *bca* gene repetitive unit (*bca* gene repetitive unit-like sequence).

We also showed that the results of PCR using two or more primer pairs that we had designed for individual genes (*rib*, *alp2*, and *alp3* genes) correlated well, supporting the specificity of each set. The only exception, as mentioned above, was ribS1/ribA3, which produced a non-specific amplicon from one of 224 isolates tested.

Example 6 - The relationship between surface protein antigen gene profiles and cps serotypes/serosubtypes.

Surface protein gene profiles.

For each gene (except *bca* gene repetitive unit or *bca* gene repetitive unit-like region), we selected two primer pairs to identify and characterise GBS surface protein by PCR. Each isolate was given a protein gene profile code according to PCR results as follows:

"A": 5'end of *bca* gene amplified by bcaS1/bcaA and bcaS2/bcaA;

"a" or "as": *bca* gene repetitive unit or *bca* gene repetitive unit-like region amplified by bcaRUS/bcaRUA, with multiple or single band amplicons, respectively;

"B": *bac* gene amplified by GBS1360S/GBS1937A and IgAagGBS/RIgAagGBS (>20 subgroups based on sequence heterogeneity).

"R": *rib* gene amplified by ribS2/ribA1 and ribS2/ribA2;

"alp2": *alp2* gene amplified by bal23S1/bal2A2 and bal23S2/bal2A1 and

"alp3": *alp3* gene amplified by bal23S1/bal3A and bal23S2/bal3A (Table 7).

Four common profiles accounted for 203 of 224 (90.6%) isolates: "R" (62 isolates), "AaB" (51 isolates), "a" (49 isolates) and "alp3" (41 isolates) (see Table 4). Only two isolates contained no surface protein gene markers. All but one isolate with the *bac* gene ("B") also had *bca* gene, with its repetitive unit ("Aa"); one had *rib* gene. All "alp2" isolates contained single *bca* repetitive unit-like sequences ("as"). "A", "R", "alp2" and "alp3" were all mutually exclusive. 62 of 63 isolates with *rib* gene ("R") and 41of 41 isolates with *alp3* gene had no other protein antigen markers.

The relationship between surface protein antigen gene profiles and cps serotypes/serosubtypes.

A *cps* molecular serotype (MS) was assigned to all isolates in accordance with the methods described in Examples 1 to 4 and the results correlated with conventional serotyping (CS) results except for 19 of 224 isolates that were nontypable using antisera. The relationship between surface protein gene profiles and cps MS are summarised in Table 9.

The following strong associations were confirmed or demonstrated between: MS Ia and *bca* gene repetitive unit or *bca* gene repetitive unit-like sequence (most with profile "a"); MS serosubtypes III-1 and III-2 and *rib* gene; MS serosubtype III-3 and *alp2* gene; MS Ib and *bca/bac* genes and MS V and *alp3* gene. MS II showed the most varied surface protein gene profiles. However, the relationships were not absolute and different combinations of *cps* serotypes and protein gene profiles produced 31 different serovariants or 51 when *bac* gene ("B") subgroups were considered.

Example 7 - The relationship between surface protein antigens and protein gene profiles.

Based on conventional serotyping, 33 isolates (belonging to CS Ia/c, Ib/c, IIc, IIb, IIIc or IIIb) reacted with the C antiserum. The surface protein gene profiles of all these isolates contained *bca* gene ("A") or *bca* gene repetitive unit-related markers ("a" or "as"): Aa, 3; AaB, 18; a, 11; alp2as,1. Twenty nine isolates reacted with the R antiserum and, of these, 22 contained *rib* gene and six, *alp3* gene. The strain used to raise the R protein antiserum (Prague 25/60) contained a presumed *rib*-like gene (see above and Figure 3).

Example 8 - Identification of mobile genetic elements suitable for molecular subtyping

We developed a series of PCR primers to screen for the presence of five mobile elements in GBS serotypes.

Specificity of primers pairs.

All the primer pairs produced amplicons of the expected lengths (Table 11) from some reference and/or some clinical isolates (Table 12). To evaluate the specificity of our primer pairs, we sequenced all amplicons produced by primers IS1548S/IS1548A3 and ISSa4S/ISSa4A2, and amplicons, selected from both

reference and clinical isolates, produced by IS861S/IS861A2 (12 isolates), IS1381S1/IS1381A (24 isolates) and GBSi1S1/GBSi1A2 (11 isolates).

All 41 IS*1548* and 15 ISSa*4* amplicon sequences were identical with the corresponding sequences in GenBank (Y14270 and AF165983, respectively). Five of 12 IS*861* amplicon sequences were identical with the corresponding IS*861* sequence in GenBank (M22449). The other seven differed, at position 732, from the published sequence (G to A) and the reference strain Prague 25/60 had two additional differences - G to A and T to A - at positions 576 and 830 of M22449, respectively.

Previously, we found a full-length insertion sequence IS*1381* (AF367974) within C beta antigen gene of a clinical isolate, with several differences compared with the original published sequence (AF064785): the terminal inverted repeats contained 15, rather than 20 base pairs (bp); there was a three bp deletion and four individual bp differences in the putative transposase pseudogene between positions 419 to 429 (of the original GenBank sequence) - **GGG ATC CGA TT** (AF064785) vs **CAG A-- -GG TA** (AF367974; our sequence). All amplicons of primer pair IS1381S1/IS1381A from 12 reference and 12 selected clinical isolates were identical with each other and with that of our IS*1381* sequence in GenBank (AF367974) but different, as above, from the original reported IS*1381* sequence (AF064785).

The amplicons of primer pair GBSi1S1/GBSi1A2 from all four GBSi1-positive reference strains and seven selected clinical isolates were sequenced. Six (including those of three reference strains) were identical with the corresponding GBSi1 sequence in GenBank (AJ292930). Amplicons from four clinical isolates showed three site-variations (C to T at position 767, A to C at position 846 and T to C at position 923 of AJ292930 sequence). The reference strain Prague 25/60 showed only the first two of these site-variations.

In addition to sequencing, we evaluated the specificity of our primer pairs by comparing PCR results for two or more primer pairs for each target (Table 11). In all cases, the same sets of isolates gave positive results when tested with PCR targeting the same mobile genetic elements, thus confirming the specificity of the primer pairs.

PCR results using specific primer pairs for all five mobile genetic elements.

IS*861*, IS*1548*, IS*1381*, ISSa*4* and GBSi1 were identified in 55%, 18%, 85%, 7% and 19% of isolates, respectively. None of the mobile elements was detected in 10 (4%) isolates. The distributions of the five mobile elements identified by PCR in the 224 GBS isolates tested in the previous examples are shown in Table 12. IS*1381*

was detected alone in 79 isolates and GBSi1 alone in one. Forty-six isolates contained two different insertion sequences (IS*861* and IS*1381*, 42 isolates ; IS*1548* and IS*1381*, three isolates; ISSa*4* and IS*1381*, one isolate). Forty-four isolates contained three (IS*861*, IS*1548* and IS*1381* 34; IS*861*, ISSa*4* and IS*1381*, 10) and one contained all four insertion sequences. Forty-one isolates contained GBSi1 in combination with one (IS*861*, 22; IS*1381*, one isolate) two (IS*861* and IS*1381*, 11; ISSa*4* and IS*1381*, three isolates) or three (IS*861*, IS*1548* and IS*1381*, four isolates) insertion sequences.

PCR results for the 194 invasive isolates using specific primer pairs for all five mobile genetic elements - .

The numbers of isolates containing different mobile genetic elements (mge) combinations (from none to four per isolate) are shown in Table 13. IS1381, IS861, IS1548, ISSa4 and GBSi1 were identified in 87%, 52%, 17%, 6% and 18% of isolates, respectively. Six (3%) isolates contained no mge.

Example 9 - The relationships between *cps* serotypes, serosubtypes, surface protein gene profiles and mobile genetic elements.

The distribution of each of the five mobile genetic elements in different *cps* serotypes, serotype III subtypes and surface protein gene profiles are shown in Tables 12 and 13. The most consistent findings for each sero/serosubtype were:

1) Serotype Ia - most (>80%) expressed proteins that closely related with C alpha protein and contained IS1381
2) Serotype Ib - most (>90%) expressed C alpha and C beta proteins and contained IS861 and IS1381
3) Serotype II - exhibited two common patterns:
   a) >50% expressed C alpha protein (and often C beta) and contained IS861, IS1381 and sometimes other mobile elements, especially ISSa4 or
   b) >25% expressed Rib protein and contained IS861, IS1381 and GBSi1
4) Serosubtype III-1 - all expressed Rib protein and contained IS861, IS1548 and IS1381 but not GBSi1.
5) Serosubtype III-2 - all expressed Rib protein and contained IS861 and GBSi1 but neither IS1548 nor IS1381.
6) Serosubtype III-3 - all expressed C alpha-like protein 2 and contained no mobile genetic elements.
7) Serosubtype III-4 - expressed various proteins; all contained GBSi1.

8) Serotype IV - most expressed proteins that closely related with C alpha protein and contained IS1381
9) Serotype V - most expressed C alpha-like protein 3 contained IS1381
10) GBSi1 and IS1548 were mutually exclusive in serotype III (III-1, III-2 and III-4) but not in serotype II.
11) All isolates that expressed C alpha-like protein 2 contained no insertion sequences.

Predominant relationships between MS/sst, pgp and mge.

Figure 5 shows the relationships between the various genetic markers. IS1381 was present in nearly all isolates of MS Ia, Ib, IV, V and VI, but in none of sst III-2 or III-3. IS1548 was found exclusively, and GBSil most commonly, in serotypes II or III; three isolates (all MS II) contained both GBSi1 and IS1548. IS861 was found in all sst III-1 and III-2 and most MS II and Ib isolates but only in 14% of other MS isolates. ISSa4 was present in only 6% of isolates, more than half of which were MS II; it was present in one invasive isolate obtained before 1996 (1994). IS1381 was found in most isolates except those in cluster 8, pgp "alp2", which had no insertion sequences. IS861was found in most genotypes with pgp "AaB" (clusters 3 and 4) and all genotypes with pgp "R" (clusters 6 and 7).

Genotypes based on MS/sst, pgp, bac subtypes and mge.

MS/sst, pgp, bac subtype (for isolates with pgp "B") and the presence of various combinations of mge provide a PCR/sequencing-based genotyping system. The 194 invasive isolates in this study represented seven serotypes, ten MS/sst, 41 subtypes based on the distributions of pgp and mge or 56 genotypes when *bac* subtypes (mainly in MS Ib) were included (Figure 5).

Theoretical GBS clonal population structure.

Theoretically there are 13 possible GBS MS/sst (eight MS - Ia, Ib, II, IV-VIII, four sst III 1-4 and cps gene cluster absent) and at least 10 pgp (none, "Aa", "AaB", "a", "as", "R", "RB", "alp2as", "alp3" or "alp4a"). If the 22 bac subgroups identified so far are included, there are up to 31 pgp. If the five mge were independently, randomly distributed and present or absent, there would be $13x31x2^5$= 12,896 different possible combinations of molecular markers. The fact that only 56 different combinations were found (Figure 5), demonstrates that markers are not randomly distributed or, in other words, these invasive Australasian GBS isolates have a clonal population structure. It

is possible, but unlikely, that these isolates represent a very limited number of GBS genotypes.

The phylogenetic relationship of Australasian invasive GBS.

The 56 genotypes formed eight clusters, separated at a genetic distance of about ~16 (or three cluster groups separated at a distance of ~22.5). The pgp was the main determinant of cluster separation (Figure 5). 94% of isolates belonged to five MS (Ia, Ib, II, III and V), 62% belonged to five (9%) genotypes (Ia-1, Ib-1, III-1, III-2, V-1) and 92% belonged to the five largest clusters (1, 2, 4, 6 and 7). Cluster group A, the largest, contained 139 (72%) isolates and 48 (86%) genotypes, 45 of which contained fewer than five isolates, whereas cluster group B contained 49 (25%) isolates and five (9%) genotypes.

The main characteristics of each cluster were as follows:

Cluster 1. "alp3", IS1381 (39 isolates, four MS, 11 genotypes; predominant genotype V-1).

Cluster 2: "a" or "as", IS1381 (55 isolates, four MS, 12 genotypes, predominant genotype Ia-1).

Cluster 3: "Aa" or "AaB", MS II, IS1381, IS 861 (10 isolates, six genotypes).

Cluster 4: "AaB", IS1381, IS861 (35 isolates, two MS: VI or Ib; 18 genotypes; predominant genotype Ib-1).

Cluster 5. "AaB", IS861, GBSi1, genotype III-4-1 (one isolate).

Cluster 6: "R", IS861 and GBSil (22 isolates, three MS/genotypes; predominant genotype III-2).

Cluster 7: "R", IS1381 and IS861 (27 isolates; two MS/genotypes; predominant genotype III-1).

Cluster 8: "alp2as", no IS (six isolates; three MS/genotypes; one contained GBSi1).

The phylogenetic study showed that the dendrogram inferred by SSPS was very robust.

The relationship between genotypes and GBS disease patterns.

The distribution of MS and genotypes in different age groups of patients with invasive GBS disease is shown in Table 14. All common MS were represented in more than one patient group. However, there were highly significant associations (when compared with all other age-groups) between sst III-2 and late onset neonatal infection (p=0.0005) and MS V and infection in the elderly (p=0.001).

There were 17 isolates from cerebrospinal fluid specimens, nine (53%) of which were MS III (from three different sst/genotypes, each in a different cluster). The other eight isolates were distributed among five MS, seven genotypes and four clusters. Meningitis occurred in all age-groups but comprised 23% of cases in the late onset neonatal group compared with 5% in all other groups.

DISCUSSION

Capsule production in GBS is controlled by capsular polysaccharide synthesis (*cps*) gene cluster, which had been sequenced for serotype Ia and serotype III before we began our study. Corresponding sequences for serotype Ib (Miyake *et al.*, 2001 submitted into GenBank, GenBank accession number: AB050723), and for serotypes IV, V, and VI (McKinnon *et al.*, 2001 submitted into GenBank, GenBank accession numbers: AF355776, AF349539, AF337958, respectively) were released recently when the project was nearly finished but those for the other three serotypes (II, VII and VIII), the sequences of *cps* gene clusters, have not been published previously.

The sequences of *cps* gene clusters for serotypes Ia, and III showed considerable homology at the 3'-end of *cpsD-cpsE-cpsF*-and the 5'-end of *cpsG*. We designed a series of primers to amplify a 2226/2217 bp segment in this region and found that amplicons were obtained from all serotypes except VIII. This confirmed a previous suggestion that serotype VIII is significantly different from other serotypes in this region.

Using eight serotype (Ia to VII) reference strains, we showed more than 50 heterogeneity points between serotypes (Figure 1, Table 4). Using 63 selected clinical isolates that had been serotyped by conventional methods, we found that these inter-serotype differences were generally consistent and specific, especially the 23 sites clustered at the 3'-end of the regions. We used these differences to assign serotypes to the remaining clinical isolates collected in this study, without knowledge of the serotype obtained by conventional methods.

Sequence analysis of the 3'-end of *cpsG-cpsH-cpsI/cpsM* for serotypes Ia, III, Ib, IV, V and VI showed that this region is highly variable (Figure 3), making this region a suitable target for direct serotype identification by PCR. We designed several pairs of MS-specific primers for MS Ia, Ib, III, IV, V and VI and used them to test two CS reference panels. Selected primer pairs were used for MS, by PCR alone, of 86.9% of our 206 clinical isolates. Using rapid-cycle MS-specific PCR, results are available within one working day. In future, it will be possible to extend this method to all MS, when *cps* gene cluster sequences in

this region are available for serotypes II, VII and VIII. Meanwhile, MS II and VII can be identified by sequencing the 790 bp PCR amplicons of the 3'-end of *cpsE-cpsF*-the 5'-end of *cpsG* (Figure 1, Table 4). A positive GBS-specific PCR and negative PCR results with all the primers that amplify the 790 bp, identified MS VIII, by exclusion.

In future, and in some laboratories currently, sequencing of the 790 bp PCR amplicons of the 3'-end of *cpsE-cpsF*-the 5'-end of *cpsG* for all isolates may be more convenient, as only one method and fewer primers are needed. However, if sequencing is not available in-house, the turn-around time is longer and a small proportion of serotypes would be wrongly assigned (serosubtypes III-3 and III-4 as MS Ia and II, respectively). This could be avoided by screening with MS III-specific PCR first. Sequencing the 790 bp PCR amplicon, allows MS III to be subtyped on the basis of the sequence heterogeneity.

Previous studies have shown that serotypes Ia, Ib, II, III, and V are those most frequently isolated from normally sterile sites, in the United States and several countries. Serotypes VI and VIII are the predominant serotypes isolated from patients in Japan, but are uncommon elsewhere. Although our isolates were selected, they were probably representative of those causing disease in Australasia; Ia, Ib, II, III, and V were the most common serotypes identified, although there were small numbers of serotypes IV,VI and, VIII.

Up to 13 % of GBS isolates are non-serotypable and in our study the proportion was 8.7% (18/206) using the antisera available. This may be due to decreased type-specific-antigen synthesis; non-encapsulated phase variation; or insertion or mutation in genes of *cps* gene clusters. One non-serotypable strain GBS in our study had a T base deletion in *cpsG* gene, which caused a change in the *cpsG* gene reading frame.

We have also developed PCR-based methods to identify GBS surface protein genes and further characterise these isolates. Using the published *bac* gene sequence, we modified *bac* gene-specific primers and designed new primers, with high melting temperatures (>70 °C) suitable for rapid cycle PCR targeting all major surface protein genes.

As previously reported, a published PCR primer pair targeting the *bca* gene repetitive unit (at the 3'-end of *bca* gene), was not entirely specific for *bca* gene. We designed two new primer pairs targeting the 5'-end of *bca* gene, to improve the specificity. However, very few serotype Ia strains gave positive results using these primers whereas all were PCR positive using primers targeting the *bca* gene repetitive unit. These results were consistent with a previous report, that a probe targeting the 5'-end of *bca* gene hybridized with only

one of nine serotype Ia strains, but a large *bca* gene probe, including the tandem repeat region, hybridized with all nine strains.

PCR specific for *rib*, *alp2* and *alp3* genes has not been described previously. The primer pairs we designed mainly targeted the 5'-ends of the gene and were chosen after comparing the gene heterogeneity with related gene sequences. We designed two or more primer pairs for each gene to check primer specificity by comparison of results of different PCR targeting the same genes. Protein gene profiles "alp2" and "alp3"were distinguished on the basis of the *alp2* and *alp3* gene -specific PCR and/or two sequence heterogeneity sites in the amplicons of bcaS1/balA, or bcaS2/ balA.

To confirm the specificity of our primers, we used them to examine two reference panels and selected GBS isolates. The longest amplicons produced by PCR for each gene were sequenced, to provide maximal sequence information and ensure that the inner primers were not located at strain heterogeneity sites. Our sequencing results confirmed the specificity of the primers. Two pairs of primers for each gene were compared, with similar results. Finally, six gene/region specific primer pairs (including the one targeting the *bca* gene repetitive unit) were used to define protein antigen gene profiles for all 224 isolates.

The study showed that only one member of the surface protein gene family containing repetitive sequences - *rib*, *bca*, *alp2*, and *alp3* genes-could be present in any single isolate. However, all isolates containing *bac* gene, which is not a member of the surface protein gene family containing repetitive sequences, also contained either *bca* gene (51/52) or *rib* gene (1/52).

Bac gene was present in 23% of isolates, a similar proportion to that (19-22%) previously reported. In common with others, we found variations in the *bac* gene due to variable small internal repetitive sequences. These *bac* gene repetitive sequences were irregular (unlike those of the *bca-rib* gene family). Their role is not clear, but they are potentially useful molecular markers for epidemiological studies.

Our data show that some serotype III isolates (our MS serosubtypes III-1 and III-2) were closely associated with *rib* gene, and others (our MS serosubtype III-3) with *alp2* gene. Serotype Ib was associated with *bca* and *bac* genes and serotype V with *alp3* gene. However, as the relationship was not absolute, different combinations of *cps* serotypes-serosubtypes/protein gene profiles identified many serovariants, which will be useful in epidemiological studies and in formulation of conjugate vaccines. Based on PCR only, we were able to divide

our 224 isolates into 31 serovariants based on *bac* gene (B) groups or 51, based on subgroups. Theoretically, there are likely to be additional serovariants.

We found that the antisera to "c" and "R" protein antigens were not entirely specific for any particular protein genes. However, reaction with "c" antiserum mostly reflected the presence of genes encoding C alpha (*bca* gene) and related protein antigens (at least including *alp2* gene) and the antiserum to "R" with those encoding Rib (*rib* gene) and related proteins (at least including *alp3* gene, and the rare presumed *rib*-like gene).

We have also investigated the presence of a number of mobile element in different serotypes of GBS. Four different insertion sequences have been identified previously in GBS. Multiple copies of IS*861* in some serotype III isolates were associated with increased capsule gene expression. We found IS*861* in all serosubtypes III-1 and III-2 and most serotype II and Ib isolates but few others. All IS*861*-containing isolates contained at least one additional mobile element.

Multiple copies of IS*1381* have been found in a high proportion GBS and other *Streptococcus* species, including *S. pneumoniae* and used as probes for restriction fragment length polymorphism (RFLP) analysis of GBS for epidemiological studies (Tamura et al., 2000). We found IS*1381* in 85% of isolates overall. They were present in all isolates of serosubtype III-1 but none of serosubtypes III-2 or III-3. Our IS*1381* sequences, from 24 isolates, were identical with each other, but differed at several sites, from that previously described (AF064785). The significance of these differences is unknown, but it emphasizes the importance of confirming sequences from as many different strains as possible.

ISSa*4* was first identified in a nonhemolytic GBS isolate, in which it caused insertional inactivation of the gene *cylB,* which is part of an ABC transporter involved in production of hemolysin. Only a small proportion of (mainly hemolytic) GBS isolates (4%) contained ISSa*4,* all of which had been isolated since 1996 and it was postulated that ISSa*4* had been newly acquired by GBS. We also found ISSa*4* in only a small proportion of isolates (7%) but it was present in similar proportions of clinical isolates obtained before (4 of 44) and during or after (11 of 162) 1996.

IS*1548* was first discovered in some hyaluronidase-negative GBS serotype III isolates, in which it caused insertional inactivation of the gene *hylB* (one of a cluster responsible for production of hyaluronidase, an important GBS virulence factor) (Granlund et al., 1998). A copy of IS*1548* is also found downstream of the C5a peptidase gene (also associated with virulence), in

isolates that contain it. Most IS*1548*-containing isolates were from patients with endocarditis and it was postulated that inactivation of hyaluronidase production and/or some effect on C5a peptidase may allow GBS isolates to adhere to and survive on heart valves.

We found IS*1548* in all serosubtype III-1 isolates, which represented 52% of 58 serotype III isolates in our collection, from superficial (eight of 12) and normally sterile (22 of 46) specimens. The latter were from neonates (seven of 20), adults (three of six) and subjects of unspecified age (12 of 20) (data not shown). Although specific clinical data were unavailable, GBS endocarditis is uncommon and likely to have been present in few, if any, of these subjects. Further study is required to elucidate the association with this insertion sequence with specific virulence factors and clinical syndromes.

We found GBSi1, a group II intron, in 19% of our 224 isolates overall; it was commonly associated with IS*861*, and the distribution varied with serotype/serosubtype. It was rarely found in serotypes other than II and III. It was present in more than 50% of serotype II isolates, including four, which also contained IS*1548*. It was found in all serosubtypes III-2 and III-4 isolates, in which IS*1548* was not found, but in no serosubtype III-1 isolates which did contain IS*1548* or serosubtype III-3 isolates which did not.

Our subdivision of GBS serotype III into four serosubtypes, based on differences within the *cps* gene cluster was supported by corresponding differences in surface protein gene profiles and distribution of the five mobile elements described in this study. Although we did not test our isolates for hyaluronidase activity, it is likely that our serosubtype III-1, which expresses Rib protein and contains IS*1548*, IS*861* and IS*1381*, corresponds with the hyaluronidase negative subtype III-2, described by Bohnsack et al., 2001. Our serosubtype III-2 also expresses Rib protein and contains IS*861* and GBSi1 and probably corresponds with subtype III-3 of Bohnsack et al., 2001. Serosubtypes III-3 and III-4 were represented by relatively few isolates. The former (in common with some serotype Ia isolates) expressed the C alpha-like protein 2 and contained no mobile elements (an otherwise uncommon finding). The latter is closely related to serotype II, with which it shares sequence homology in a section of the cps gene cluster and various surface protein profiles and mobile elements.

Summary

Our aim has been to develop a comprehensive genotyping system for group B streptococcus (GBS). Such a system should ideally be reproducible, objective and transportable between laboratories, comparable with and complementary to other typing methods and able to incorporate known virulence markers. Based on these criteria, we first developed a molecular serotyping (MS) method based on the *cps* gene cluster. It compared favourably with, but was more sensitive than, conventional serotyping (CS) and allowed us to identify several subtypes of serotype (sst) III, as described by others. We have also developed a second molecular subtyping method based on the family of genes encoding variable surface protein antigens (*bca/rib/alp2/alp3/alp4*) and the IgA binding protein C beta (*bac*), is more sensitive and objective than conventional protein serotyping, which cannot type all isolates and is sometimes misleading. Our methods also can identify more members of the family of variable antigen genes and distinguish numerous *bac* subgroups. A third subtyping method uses five mobile genetic elements (mge) including four different insertion sequences (IS) and a type II intron, which have been identified in GBS. The use of this third method further enhances the discriminatory ability of our genotyping system.

We then used our typing system to examine the population genetic structure and age-related disease distribution of genotypes among 194 invasive GBS isolates.

We used mainly invasive GBS isolates to demonstrate the practical value of our genotyping system, confirm their clonal population structure and determine the distribution of genotypes in different patient groups. The isolates originated from patients of all ages with GBS sepsis. About half were consecutive GBS isolates from blood or CSF, at a large diagnostic laboratory in a general adult hospital, with an obstetric unit (i.e there were no isolates from children other than neonates). The rest were consecutive isolates referred for serotyping from all over New Zealand. Thus the overall age distribution is representative of that in the population affected by GBS disease, except that children beyond the early neonatal period are probably under-represented. However, the distribution of genotypes within each age-group should be representative.

Among our 194 Australasian invasive GBS isolates we identified 56 genotypes, of which five (Ia-1, Ib-1, III-1, III-2 and V-1) accounted for 62% of isolates.

The phylogenetic tree derived from our results showed relationships between *cps* serotype and protein gene profiles (pgp). Our results also show that certain known virulence markers – C beta, C alpha variants and hyaluronidase production (indirectly) - were associated with distinct clonal lineages.

Our genotyping system, based on three sets of genetic markers, is highly discriminatory. Because it provides useful phenotypic data, including antigenic composition, it will be useful for epidemiological surveillance of GBS, especially in relation to potential GBS vaccine use. Study of the relationships between putative high-virulence genotypes and patient characteristics (age and/or underlying risk factors), and whether there are significant differences between CSF isolates (or genotypes) and other invasive or colonising strains, will be facilitated by our genotyping system. Using this system, we have demonstrated a clonal population structure among invasive Australasian GBS isolates. This system will be applied to colonising GBS isolates, to identify markers of virulence.

Thus, we have developed an alternative to conventional serotyping for GBS, which is accurate and reproducible, can be performed by any laboratory with access to PCR/sequencing and, importantly, does not require panels of serotype-specific antisera that are increasingly difficult to maintain. All isolates are serotypable and sequencing of a relatively limited 790 bp region can provide additional serosubtyping information for MS III. The molecular methods we have described for serotype identification, together with the protein profiling (or protein antigen subtyping) and identification of mobile genetic elements (or mobile genetic elements subtyping) provide potentially useful markers for further phylogenetic and epidemiological studies of GBS as well as comprehensive strain identification that will be useful for epidemiological and other related studies that will be needed to monitor GBS isolates before and after introduction of GBS conjugate vaccines.

The various features and embodiments of the present, referred to in individual sections above apply, as appropriate, to other sections, mutatis mutandis. Consequently features specified in one section may be combined with features specified in other sections, as appropriate.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are readily apparent to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

REFERENCES

Ahmet, Z., P. Stanier, D. Harvey, and D. Holt. 1999. New PCR primers for the sensitive detection and specific identification of group B beta-hemolytic streptococci in cerebrospinal fluid. Mol. Cell. Probes. **13:**349-357.

Arakere, G., A.E. Flores, P. Ferrieri, and C.E. Frasch. 1999. Inhibition enzyme-linked immunosorbent assay for serotyping of group B streptococcal isolates. J. Clin. Microbiol. **37:**2564-2567.

Bohnsack, J. F., S. Takahashi, S. R. Detrick, L. R. Pelinka, L. L. Hammitt, A. . Aly, A. A. Whiting, and E. E. Adderson. 2001. Phylogenetic Classification of Serotype III Group B Streptococci on the Basis of hylB Gene Analysis and DNA Sequences Specific to Restriction Digest Pattern Type III-3. J. Infect. Dis. **183:**1694-1697.

Cropp, C.B., R.A. Zimmerman, J. Jelinkova, A.H. Auernheimer, R.A. Bolin, and B.C. Wyrick. 1974. Serotyping of group B streptococci by slide agglutination fluorescence microscopy, and microimmunodiffusion. J. Lab. Clin. Med. **84:**594-603.

Granlund, M., L. Oberg, M. Sellin, and M. Norgren. 1998. Identification of a novel insertion element, IS*1548*, in group B streptococci, predominantly in strains causing endocarditis. J. Infect. Dis. **177:**967-976

Hakansson, S., L.G. Burman, J. Henrichsen, and S.E. Holm. 1992. Novel coagglutination method for serotyping group B streptococci. J. Clin. Microbiol. **30:**3268-3269.

Harrison, L.H., J.A. Elliott, D.M. Dwyer, J.P. Libonati, P. Ferrieri, L. Billmann, and A. Schuchat. 1998. Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. Maryland Emerging Infections Program. J. Infect. Dis. **177:**998-1002.

Hassan, A.A., A. Abdulmawjood, A.O. Yildirim, K. Fink, C. Lammler, and R. Schlenstedt. 2000. Identification of streptococci isolated from various sources by determination of cfb gene and other CAMP-factor genes. Can. J. Microbiol. **46:**946-951.

Hickman, M.E., M.A. Rench, P. Ferrieri, and C.J. Baker. 1999. Changing epidemiology of group B streptococcal colonization. Pediatrics. **104:**203-209.

Holm, S.E., and S. Hakansson. 1988. A simple and sensitive enzyme immunoassay for determination of soluble type-specific polysaccharide from group B streptococci. J. Immunol. Methods. **106**:89-94.

Ke, D., C. Menard, F.J. Picard, M. Boissinot, M. Ouellette, P.H. Roy, and M.G. Bergeron. 2000. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin. Chem. **46**:324-331.

Kong, F., X. Zhu, W. Wang, X. Zhou, S. Gordon, and G.L. Gilbert. 1999. Comparative analysis and serovar-specific identification of the multiple banded antigen genes of *Ureaplasma urealyticum* biovar one. J. Clin. Microbiol. **37**: 538-543.

Kong, F., S. Gordon, and G.L. Gilbert. 2000. Rapid-Cycle PCR for Detection and Typing of *Mycoplasma pneumoniae* in Clinical Specimens. J. Clin. Microbiol. **38**:4256-4259.

Maeland, J. A., O. G. Brakstad, L. Bevanger, and A. I. Kvam. 1997. *Streptococcus agalactiae* beta gene and gene product variations. J. Med. Microbiol. **46**:999-1005.

Maeland, J. A., O. G. Brakstad, L. Bevanger, and S. Krokstad. 2000. Distribution and expression of bca, the gene encoding the c alpha protein, by *Streptococcus agalactiae*. J. Med. Microbiol. **49**:193-198.

Mawn, J.A., A.J. Simpson, and S.R. Heard. 1993. Detection of the C protein gene among group B streptococci using PCR. J. Clin. Pathol. **46**:633-636.

Nagano, Y., N. Nagano, S. Takahashi, K. Murono, K. Fujita, F. Taguchi, and Y. Okuwaki. 1991. Restriction endonuclease digest patterns of chromosomal DNA from group B beta-haemolytic streptococci. J. Med. Microbiol. **35**:297-303.

Rolland, K., C. Marois, V. Siquier, B. Cattier, and R. Quentin. 1999. Genetic features of *Streptococcus agalactiae* strains causing severe neonatal infections, as revealed by pulsed-field gel electrophoresis and hylB gene analysis. J. Clin. Microbiol. **37**:1892-1898.

Tamura, G. S., M. Herndon, J. Przekwas, C. E. Rubens, P. Ferrieri, and S. L. Hillier. 2000. Analysis of restriction fragment length polymorphisms of the insertion sequence IS*1381* in group B Streptococci. J. Infect. Dis. **181**:364-368.

Triscott, M.X., and G.H. Davis. 1979. A comparison of four methods for the serotyping of group B streptococci. Aust. J. Exp. Biol. Med. Sci. **57**:521-527.

Wilkinson, H.W., and M.D. Moody. 1969. Serological relationships of type I antigens of group B streptococci. J. Bacteriol. **97**:629-34.

Zuerlein, T.J., B. Christensen, and R.T. Hall. 1991. Latex agglutination detection of group-B streptococcal inoculum in urine. Diagn. Microbiol. Infect. Dis. **14**:191-194.

Table 1. GBS reference panels used in this study.

Lab strain number	Source	Serotype	MS/ serosubtype	GenBank accession numbers
Reference panel 1[1]				
090	Channing	Ia	Ia	AF332893
H36B	Channing	Ib	Ib	AF332903
18RS21	Channing	II	II	AF332905
M781	Channing	III	III-2[3]	AF332896
3139	Channing	IV	IV	AF332908
CJB 111	Channing	V	V	AF332910
SS1214	Channing	VI	VI	AF332901
7271	Channing	VII	VII	AF332913
JM9 130013	Channing	VIII	VIII	
Reference panel 2[2]				
NZRM 908 (NCDC SS615)	ESR	Ia	Ia	AF332894
NZRM 909 (NCDC SS618)	ESR	Ib	Ib	AF332904
NZRM 910 (NCDC SS700)	ESR	Ic	Ia	AF332914
NZRM 911 (NCDC SS619)	ESR	II	II	AF332906
NZRM 912 (NCDC SS620)	ESR	III	III-3[3]	AF332897
NZRM 2217 (Prague 25/60)	ESR	Non-typable (R)	II	AF332907
NZRM 2832 (Prague 1/82)	ESR	IV	IV	AF332909
NZRM 2833 (Prague 10/84)	ESR	V	V	AF332911
NZRM 2834 (Prague 118754)	ESR	VI	VI	AF332902

Notes.

1. Reference panel 1: supplied by Dr Lawrence Paoletti, Channing Laboratory, Boston, USA.
2. Reference panel 2: New Zealand Reference Medical Culture Collection strains supplied by Dr Diana Martin, ESR, Porirua, Wellington, New Zealand.
3. MS III serosubtypes based on sequence heterogeneity; see text for more detail

Table 2. Oligonucleotide primers used in this study.

Primer	Target gene	Tm °C [1]	GenBank accession numbers	Sequence [2-4]
CFBS	*cfb*	56.7	X72754	**328**GAT GTA TCT ATC TGG AAC TCT AGT G**352**
Sag59[5]	*cfb*	77.4	X72754	**350**GTGGCTGGTGCATTGTTAT TTT CAC CAG CTG TAT TAG AAG TA**391**
Sag190[5]	*cfb*	76.8	X72754	**545**CATTAACCGGTTTTTCATAATCT GTT CCC TGA ACA TTA TCT TTG AT**500**
CFBA	*cfb*	63.2	X72754	**568**TTT TTC CAC GCT AGT AAT AGC CTC**545**
16SS	16S rRNA	69.3	AB023574	**1441**GCC GCC TAA GGT GGG ATA GAT G**1462**
23SA	23S rRNA	65.7	X68427	**70**CGT CGT TTG TCA CGT CCT TC**51**
DSF2[6]	16S rRNA	75.9	AB023574	**975**CATCCTTCTGACC GGC CTA GAG ATA GGC TTT CT**1007**
DSR1[6]	16S rRNA	81.5	AB023574	**1250**CGTCACCGG CTT GCG ACT CGT TGT ACC AA**1222**
cpsDS	*cpsD*	69.1	AB028896 (Ia), AF163833 (III)	**4892/4593**GCA AAA GAA CAG ATG GAA CAA AGT GG**5007/4618**
cpsES	*cpsE*	65.7	AB028896 (Ia), AF163833 (III)	**5300/4910**CTT TTG GAG TCG TGG CTA TCT TG**5322/4932**
cpsEA1	*cpsE*	65.4	AB028896 (Ia), AF163833 (III)	**5431/5041**GA/T/GA AAA AAG GAA AGT CGT GTC G/ATT G**5612/5017**

cpsES1	*cpsE*	65.9	AB028896 (Ia), AF163833 (III)	**5612/5222**CTT GGA C/TTC CTC TGA AAA GGA TTG**5635/5245**
cpsEA2	*cpsE*	66.8	AB028896 (Ia), AF163833 (III)	**5723/5333**AAA A/CGC TTG ATC AAC AGT TAA GCA GG**5698/5308**
cpsES2	*cpsE*	70.2	AB028896 (Ia), AF163833 (III)	**6012/5622**GAT GGT/C GGA CCG GCT ATC TTT TCT C**6036/5646**
cpsEA3	*cpsE*	63.7	AB028896 (Ia), AF163833 (III)	**6116/5726**CTT AAT TTG TTC TGC ATC TAC TCG C**6092/5702**
cpsES3	*cpsE*	71.5	AB028896 (Ia), AF163833 (III)	**6410/6020**GTT AGA TGT TCA ATA TAT CAA TGA ATG GTC TAT TTG GTC AG**6450/6060**
cpsEFA	*cpsE/F* spacer	62.1	AB028896 (Ia), AF163833 (III)	**6526/6136**CCT TTC AAA CCT TAC CTT TAC TTA GC**6501/6111**
cpsFS	*cpsF*	75.0	AB028896 (Ia), AF163833 (III)	**6777/6387**CAT CTG GTG CCG CTG TAG CAG TAC CAT T**6804/6414**
cpsFA	*cpsF*	73.2	AB028896 (Ia), AF163833 (III)	**6859/6469**GTC GAA AAC CTC TAT A/GT A AAC/T GGT CTT ACA A/GCC AAA TAA CTT ACC**6819/6425**
cpsGA	*cpsG*	54.7	AB028896 (Ia), AF163833 (III)	**7162/6772**AAG/C AGT TCA TAT CAT CAT ATG AGA G **7138/6748**
cpsGA1	*cpsG*	74.5	AB028896 (Ia), AF163833 (III)	**7199/6809**CCG CCA/G TGT GTG ATA ACA ATC TCA GCT TC**7171/6781**
cpsGS	*cpsG*	72.24	AB028896 (Ia), AF163833 (III)	**7145/6755**ATG ATG ATA TGA ACT CTT ACA TGA AAG AAG CTG AGA TTG **7183/6793**
cpsGS1	*cpsG*	71.62	AB028896 (Ia), AF163833 (III)	**7155/6765**GAA CTC TTA CAT GAA AGA AGC TGA GAT TGT TAT CAC AC **7192/6802**

IacpsHS	*cpsH*	73.6	AB028896 (Ia)	**7698**CAT TCT TTG TTT AAA AA/CT CCT GAT TTT GAT AGA ATT TTA GCA GC**7741**
IacpsHA	*cpsH*	75.2	AB028896 (Ia)	**7993**GAA TAT TCA AAA AAT CCC ATT GCT CTT TGA GTA TGC ATA CC**7953**
IacpsHA1	*cpsH*	66.4	AB028896 (Ia)	**8271**GTA AGT TAT CAA AAT ATA ACA TCA TTA CTA TTA CTA GTA GAA ACG G**8226**
IacpsHS1	*cpsH*	77.9	AB028896 (Ia)	**8463**GGC CTG CTG GGA TTA ATG AAT ATA GTT CCA GGT TTG C**8499**
IacpsHA2	*cpsH*	58.5	AB028896 (Ia)	**8499**GCA AAC CTG GAA CTA TAT TCA T**8478**
IbcpsHS0	*cpsH*	58.6	AB050723 (Ib)	**3013**ATT GCT GCA TTC AAT TCA C**3031**
IbcpsHS	*cpsH*	81.9	AB050723 (Ib)	**3016**GCT GCA TTC AAT TCA CTG GCA GTA GGG GTT GTG TCC**3051**
IbcpsHA	*cpsH*	67.7	AB050723 (Ib)	**3297**GAT AGT TAA GGG TAT TAT AAG ATT TGA ATA TTC AAA GAA AGC**3256**
IbcpsHS1	*cpsH*	74.1	AB050723 (Ib)	**3546**TTT GGT GAG CAT ATA TAA TAG AAT AAT CAA TTT GCG GTC G**3585**
IbcpsHS2	*cpsH*	73.7	AB050723 (Ib)	**3740**CTG GCC TAT TTG GAC TAA TAA ATG TGA TTT TAG GTT TGT TTC**3781**
IbcpsHA01	*cpsH*	57.7	AB050723 (Ib)	**3781**GAA ACA AAC CTA AAA TCA CAT TTA**3758**

IbcpsHA1	*cpsH*	78.5	AB050723 (Ib)	**3894**GGC GCC ATC AAT ATC TTC AAG TGC AAA AAA TGA AAA TAG G**3855**
IbcpsIA	*cpsI*	78.2	AB050723 (Ib)	**4086**CTA TCA ATG AAT GAG TCT GTT GTA GGA CGG ATT GCA CG**4049**
IbcpsIS	*cpsI*	71.1	AB050723 (Ib)	**4116**GAT AAT AGT GGA GAA ATT TGT GAT AAT TTA TCT CAA AAA GAC G**4158**
IbcpsIA1	*cpsI*	78.6	AB050723 (Ib)	**4638**CCT GAT TCA TTG CAG AAG TCT TTA CGA TGC GAT AGG TG**4601**
IIIVIcpsHS	*cpsH*	75.3	AF163833 (III), AF337958 (VI)	**7275/7120**CAA GAG GAT ATA ACG TTT CAG CGA TTT ATT GCT GAG C**7311/7156**
IIIcpsHS	*cpsH*	72.1	AF163833 (III)	**7672**GAA TAC TAT TGG TCT GTA TGT TGG TTT TAT TAG CAT CGC**7710**
IIIcpsHA	*cpsH*	71.0	AF163833 (III)	**7817**GTT ATA AGA AAA ACA AGCGGT GAT AAA TAA GAA AGT CAT ACC**7776**
IVcpsHS	*cpsH*	74.1	AF355776 (IV)	**7552**CCG TAC ATA CAA CTG TTC TTG TTA GCA TTT ACT TTT CTT TGC**7593**
IVcpsHS1	*cpsH*	71.2	AF355776 (IV)	**7887**CCC AAG TAT AGT TAT GAA TAT TAG TTG GAT GGT TTT TGG**7925**
IVcpsHA	*cpsH*	77.3	AF355776 (IV)	**7951**CAT CTA CAC CCC CAC AAA ATA TTT TCC CAA AAA CCA TC**7914**
IVcpsHA1	*cpsH*	58.7	AF355776 (IV)	**7958**TGT AAA TCA TCT ACA CCC CC **7939**

IVcpsMA	*cpsM*	80.7	AF355776 (IV)	**8265**GGG TCA ATT GTA TCG TCG CTG TCA ACA AAA CCA ATC AAA TC**8225**
VcpsHS	*cpsH*	76.3	AF349539 (V)	**6943**GGG TTT AGG CGA GGG AAA CTC AGC TTA CAA AAT AGT G**6979**
VcpsHS1	*cpsH*	72.2	AF349539 (V)	**7258**CAA TTT TTA TAG GGA TGG ACA ATT TAT TCT GAG AAG TGA C**7297**
VcpsHA	*cpsH*	71.1	AF349539 (V)	**7291**TCT CAG AAT AAA TTG TCC ATC CCT ATA AAA ATT GAC ATA C**7252**
VcpsHS02	*cpsH*	59.0	AF349539 (V)	**7616**GAT GTT CTT TTA ACA GGT AGA TTA CAC**7642**
VcpsHA1	*cpsH*	66.8	AF349539 (V)	**7658**GTT GTA AAT GAG CAT AGT GTA ATC TAC CTG TTA AAA GAA C**7619**
VcpsHS2	*cpsH*	74.0	AF349539 (V)	**7871**CCC AGT GTG GTA ATG AAT ATT AGT TGG CTA GTT TTT GG**7908**
VcpsHA2	*cpsH*	58.6	AF349539 (V)	**7945**CTT TTT TAT AGG TTC GAT ACC ATC**7922**
VcpsMA	*cpsM*	73.1	AF349539 (V)	**8244**CCC CCC ATA AGT ATA AAT AAT ATC CAA TCT TGC ATA GTC AG**8204**
VIcpsHS	*cpsH*	76.7	AF337958 (VI)	**7478**CAC TAT TCC TAG TTT TTT GTG CAT ATT TGA CAG GGG CAA G**7517**
VIcpsHA	*cpsH*	76.7	AF337958 (VI)	**7517**CTT GCC CCT GTC AAA TAT GCA CAA AAA ACT AGG AAT AGT G**7478**

VIcpsHS1	*cpsH*	77.2	AF337958 (VI)	**7767**CCT TAT TGG GCA AGG TAT AAG AGT TCC CTC CAG TGT G**7803**
VIcpsHA1	*cpsH*	77.2	AF337958 (VI)	**7804**CCA CAC TGG AGG GAA CTC TTA TAC CTT GCC CAA TAA G**7768**
VIcpsIA	*cpsI*	74.5	AF337958 (VI)	**8126**GAA GCA AAG ATT CTA CAC AGT TCT CAA TCA CTA ACT CCG**8088**
cpsIA	*cpsI*	70.3	AB028896 (Ia), AF163833 (III)	**8816/8312**GTA TAA CTT CTA TCA ATG GAT GAG TCT GTT GTA GTA CGG**8778/8274**

Notes.

1. The primer Tm values are provided by the primer synthesiser (Sigma-Aldrich).
2. Numbers represent the numbered base positions at which primer sequences start and finish (numbering start point "1" refer to the start points "1" of correspondent gene GenBank accession numbers).
3. Underlined sequences show bases added to modify previously published primers.
4. Letters behind "/" indicate alternative nucleotides in different serotypes.
5. Ke *et al.*, 2000.
6. Ahmet *et al.*, 1999

Table 3. Specificity and expected lengths of amplicons of using different oligonucleotide primer pairs.

Primer pairs*	Specificity	Length of amplicons (base pairs)
Sag59/Sag190[a]	GBS (*S. agalactiae*)	196
CFBS/CFBA	GBS (*S. agalactiae*)	241
16SS/23SA	GBS (*S. agalactiae*)	433
DSF2/DSR1[a]	GBS (*S. agalactiae*)	276
cpsDS/cpsEA1	serotypes Ia to VII	449/458
cpsES/cpsEA2	serotypes Ia to VII	424
cpsES1/cpsEA3	serotypes Ia to VII	505
cpsES2/cpsEFA	serotypes Ia to VII	515
cpsES3/cpsFA[b]	serotypes Ia to VII	450
cpsFS/cpsGA1[b]	serotypes Ia to VII	423
cpsES3/cpsGA1[b]	serotypes Ia to VII	790
cpsGS/cpsIA	serotypes Ia and III	1672/1558
cpsGS1/cpsIA	serotypes Ia and III	1662/1548
cpsGS/IacpsHA1	serotype Ia	1127
cpsGS1/IacpsHA1	serotype Ia	1117
IacpsHS/IacpsHA	serotype Ia	296
IacpsHS/IacpsHA1	serotype Ia	574
IacpsHS1/cpsIA[c]	serotype Ia	354
cpsGS/IbcpsHA1	serotype Ib	1468
cpsGS1/IbcpsHA1	serotype Ib	1458
cpsGS/IbcpsIA	serotype Ib	1660
cpsGS1/IbcpsIA	serotype Ib	1650
IbcpsHS/IbcpsHA	serotype Ib	282
IbcpsHS1/IbcpsHA1	serotype Ib	349
IbcpsHS2/IbcpsIA	serotype Ib	347
IbcpsIS/IbcpsIA1[c]	serotype Ib	523
cpsGS/IIIcpsHA	serotype III	1063
cpsGS1/IIIcpsHA	serotype III	1053
IIIVIcpsHS/IIIcpsHA	serotype III	543
IIIcpsHS/cpsIA[c]	serotype III	641
cpsGS/IVcpsHA	serotype IV	1372
cpsGS1/IVcpsHA	serotype IV	1362
cpsGS/IVcpsMA	serotype IV	1686

cpsGS1/IVcpsMA	serotype IV	1676
IVcpsHS/IVcpsHA	serotype IV	400
IVcpsHS1/IVcpsMA[c]	serotype IV	379
cpsGS/VcpsHA1	serotype V	1096
cpsGS1/VcpsHA1	serotype V	1086
cpsGS/VcpsMA	serotype V	1682
CpsGS1/VcpsMA	serotype V	1672
VcpsHS/VcpsHA	serotype V	349
VcpsHS1/VcpsHA1	serotype V	401
VcpsHS2/VcpsMA[c]	serotype V	374
IIIVIcpsHS1/VIcpsHA	serotype VI	398
cpsGS/VIcpsHA1	serotype VI	1205
cpsGS1/VIcpsHA1	serotype VI	1195
cpsGS/VIcpsIA	serotype VI	1527
cpsGS1/VIcpsIA	serotype VI	1517
VIcpsHS/VIcpsHA1[c]	serotype VI	327
VIcpsHS1/VIcpsIA	serotype VI	360

Notes.

*See Table 2 for primer sequences and Figure 1 for some primer sites.

Primers used in Algorithm for molecular serotype identification-Figure 2

a. to identify GBS, b. for sequencing, c. for MS-specific PCR

Table 4. The heterogeneity of 8 GBS serotypes in the regions of the 3'-end of *cpsD-cpsE-cpsF*-and the 5'-end of *cpsG*.

Sites	Ia	Ib	II/III-4	III	IV	V	VI	VII	Specificity
***cpsD* gene**									
62	G	A	G[4]	A	A	A	A	G	Ia, II, VII
78-86	- Ia-2[1];	-	- II-2[2, 4];	- III-2[3];	+	+	-	+	See text
repetitive sequence	+ Ia-1[1]		+ II-1[2]	+ III-1[3],					
- TTACGGCGA				III-3[3]					
***cpsD/cpsE* genes**									
spacer									
138	G	G	G	G	G	A[5]	G	G	V
139	G	G	G	A III-2;	G	G	G	G	III-2
				G III-1, III-3					
144	T	T	T	G III-2;	T	T	T	T	III-2
				T III-1, III-3					
***cpsE* gene**									
198	A	C	A[4]	A	C	C[5]	A	A	Ib, IV, V
204	G	G	G	A III-2, III-3;	G	G	G	G	III-2, III-3
				G III-1					
211	T	T	T	T	T	T	G	T	VI
218	C	C	C	C	C	C	T	C	VI
240	T	T	T	T	T	T	C	T	VI

249	T	C	T[4]	T	C	C[5]	T	T	Ib, IV, V
300	C	C	C	T III-2; C III-1, III-3	C	C	C	C	III-2
321	C	C	C	T III-1; C III-2, III-3	C	C	C	C	III-1
419	T	C	T[4]	T	T	T	T	T	Ib
429	A	T	A[4]	T	T	T	T	A	Ia, II, VII
437	C	C	C; T III-4	C	C	C	C	T	VII, III-4
457	T	A	C[4]	A	A	A	A	C	Ia, II, VII
466	G	G	G	G	A	G	G	A	IV
486	G	A	A	G III-3; A III-2, III-1	A	A	A	A	Ia, III-3
602	G	G	A[4]	G	G	G	G	A	II, VII
606	T	T	T	T	T	T	C	T	VI
627	T	C	C	C	C	C	C	C	Ia
636	C	T	T	C III-1; T III-2, III-3	T	T	T	T	Ia, III-1
645	C	T	C[4]	C	T	T	C	C	Ib, IV, V
803	A	A	A	A	A	A	T	A	VI
971	C	T	T	C	C	C	T	T	Ia, III, IV, V
1026	A	G	G	G III-2, III-1; A III-3	A	A	G	G	Ia, III-3, IV, V
1044	T	T	T	T	T	T	C	T	VI

1173	A	G	A	A	A	A	A	A	Ib
1194	C	C	C	A	A	C	A	C	III, IV, VI
1251	G	G	G	G	G	G	A	G	VI
1278	A	A	A	A	A	G	A	A	V
1413	C	T	T	C III-3; T III-2, III-1	T	T	T	T	Ia, III-3
1495	C	C	C	C	C	C	A	C	VI
1500	A	G	A	A	A	A	A	A	Ib
1501	C	C	T	C	C	C	C	T	II, VII
1512	C	T	C	T III-2, III-1; C III-3	C	T	T	C	Ia, II, III-3, IV, VII
1518	T	C	T	C III-2, III-1; T III-3	T	C	C	T	Ia, II, III-3, IV, VII
1527	T	A	A	T III-3; A III-2, III-1	T	A	A	A	Ia, III-3, IV
***cpsF* gene**									
1595	T	C	T	T	T	T	C	T	Ib, VI
1611	C	C	C	C	C	C	C	T	VII
1620	C	C	C	C	C	C	C	T	VII
1627	G	G	G	G	T	G	G	G	IV
1629	G	G	G	A III-1; G III-2, III-3	G	G	G	G	III-1
1655	C	T	C	C	C	C	C	C	Ib
1832	C	C	C	C	T	C	C	C	IV

1856	T	C	T	T	T	T	T	T	Ib
1866	G	G	G	G	G	G	G	A	VII
1871	T	T	T	T	T	C	T	T	V
1892	A	A	A	A	A	G	A	A	V
1971	G	G	G	G	G	G	A	G	VI
***cpsG* gene**									
2026	G	A	G	G	G	G	G	G	Ib
2088	G	G	G	G	A	G	G	G	IV
2134	T	T	T	C III-2, III-1; T III-3	T	T	T	T	III-2, III-1
2187	C	C	C	C	C	C	C	G	VII
2196	A	A	A	A	A	A	A	G	VII

Notes.

1. Repetitive sequence: serosubtype Ia-1 present (+); serosubtype Ia-2 absent (-) (see text).
2. Repetitive sequence: serosubtype II-1 present (+); serosubtype II-2 absent (-) (see text).
3. Repetitive sequence: serosubtypes III-1 and III-3 present (+); serosubtype III-2 absent (-); serosubtype III-4 variable (see text)
4. One CS II strain has mutations at the 9 sites (see text).
5. At positions 138, 198, and 249, one CS V reference strain (Prague 10/84) is identical with corresponding sequence in GenBank (GenBank accession number AF349539), the sequences are G, A and T, respectively; another CS V reference strain (CJB 111) and all the other sequenced CS V strains are identical, the sequences are A, C and C, respectively.

Table 5. Comparison of the results of conventional serotyping (CS) and molecular serotype identification (MS)/subtyping of 206 clinical GBS isolates.

	MS/serosubtype										
CS	**Ia**	**Ib**	**II**	**III-1[1]**	**III-2[1]**	**III-3[1]**	**III-4[1]**	**IV**	**V**	**VI**	**VIII**
Ia	38										
Ib		30									
II			25								
III				27	20	4	3				
IV								7			
V									31		
VI										2	
VIII											1
NT[1]	2	5	1	3	1				5	1	
Total (206)[2]	40	35	26[2]	30	21[2]	4	3	7	36	3	1

Notes.

1. For details of MS III serosubtypes see text.

2. One mixed culture was included as two separate isolates (one serotype II, one subtype III-2).

Table 6. Oligonucleotide primers used in this study.

Primer	Target gene	Tm °C [1]	GenBank Accession numbers	Sequence [2, 3]
IgAagGBS[5]	*bac*	73.8	X59771	**2663**GCGATTAAACAA CAA ACT ATT TTT GAT A TTG ACA ATG CAA**2702**
IgAS1[4]	*bac*	72.8	X59771	**2765**GCT AAA TTT CAA AAA GGT CTA GAG ACA AAT ACG CCA G**2801**
IgAA1[4]	*bac*	78.9	X59771	**3157**CCC ATC TGG TAA CTT CGG TGC ATC TGG AAG C**3127**
RigAagGBS[5]	*bac*	76.3	X59771	**3284**CAGCCAACTCTTTC GTC GTT ACT TCC TTG AGA TGT AAC**3247**
GBS1360S[6]	*bac*	72.3	X59771	**1325**GTGAAATTGTAT AAG GCT ATG AGT GAG AGC TTG GAG**1360**
GBS1717S[4]	*bac*	75.0	X59771	**1685**ACA GTC ACA GCT AAA AGT GAT TCG AAG ACG ACG**1717**
GBS1937A[6]	*bac*	75.9	X59771	**1976**CCGTTTTAGAATCTTT CTG CTC TGG TGT TTT AGG AAC TTG**1937**
BcaRUS[7]	*bca* repetitive unit	73.5	M97256	**769**GATAAATATGATCCAA CAG GAG GGG AAA CAA CAG TAC**805**
BcaRUA[7]	*bca* repetitive unit	77.2	M97256	**1003**CTGGTTTTGGTGTCACAT GAA CCG TTA CTT CTA CTG TAT CC**963**

bcaS1[4]	*bcalalp2/alp3*	71.7	M97256 and AF291065	**208/533**GGT AAT CTT AAT ATT TTT GAA GAG TCA ATA GTT GCT GCA TCT AC**251/576**
bcaS2[4]	*bcalalp2/alp3*	78.0	M97256 and AF291065	**256/581**CCAGGGA GTG CAG CGA CCT TAA ATA CAA GCA TC**288/613**
bcaS[4]	*bca*	58.9	M97256	**370**GTT TTA GAA CAA GGT TTT ACA GC**392**
balS[4]	*alp2/alp3*	73.8	AF291065	**677**GAT CCT CAA AAC CTC ATT GTA TTA AAT CCA TCA AGC TAT TC**717**
bcaA[4]	*bca*	74.2	M97256	**597**CGTTCTAACTT CTT CAA TCT TAT CCC TCA AGG TTG TTG**560**
balA[4]	*alp2/alp3*	73.6	AF291065	**978**CCA GTT AAG ACT TCA TCA CGA CTC CCA TCA C**948**
bal23S1[4]	*alp2/alp3*	70.9	AF208158 and AF291065	**1093/1373**CAG ACT GTT AAA GTG GAT GAA GAT ATT ACC TTT ACG G**1129 /1409**
bal23S2[4]	*alp2/alp3*	72.9	AF208158 and AF291065	**1174/1454**CTT AAA GCT AAG TAT GAA AAT GAT ATC ATT GGA GCT CGT G**1213/1493**
bal2S[4]	*alp2*	59.2	AF208158	**1363**GTT CTT CCG CCA GAT AAA ATT AAG**1386**
bal2A[4]	*alp2*	58.3	AF208158	**1576**CTG TTG ACT TAT CTG GAT AGG TC**1554**
bal2A1[4]	*alp2*	78.3	AF208158	**1426**CGT GTT GTT CAA CAG TCC TAT GCT TAG CCT CTG GTG**1391**
bal2A2[4]	*alp2*	70.8	AF208158	**1518**GGT ATC TGG TTT ATG ACC ATT TTT CCA GTT ATA CG**1484**

bal3S[4]	*alp3*	57.1	AF291065	**1643**GTT CTT CCG CTT AAG GAT AGC A**1664**
bal3A[4]	*alp3*	79.2	AF291065	**1693**GAC CGT TTG GTC CTT ACC TTT TGG TTC GTT GCT ATC C**1657**
#ribS1[4]	*rib*	65.2	U58333	**216**TAC AGA TAC TGT GTT TGC AGC TGA AG**241**
ribS2[4]	*rib*	73.0	U58333	**238**GAAGTAATTTCAG GAA GTG CTG TTA CGT TAA ACA CAA ATA TG**279**
ribA1[4]	*rib*	78.8	U58333	**431**GAA GGT TGT GTG AAA TAA TTG CCG CCT TGC CTA ATG**396**
ribA2[4]	*rib*	72.6	U58333	**462**AAT ACT AGC TGC ACC AAC AGT AGT CAA TTC AGA AGG**427**
#ribA3[4]	*rib*	61.3	U58333	**570**CAT CTA TTT TAT CTC TCA AAG CTG AAG**554**

Notes.

#For sequencing use only, not entirely specific for rib gene.

1. The primer Tm values are provided by the primer synthesiser (Sigma-Aldrich).
2. Numbers represent the numbered base positions at which primer sequences start and finish (numbering start point "1" refer to the start point "1" of corresponding GenBank accession number, of which there are two for some sequences).
3. Underlined sequences show bases added to modify previously published primers.
4. Primers designed by us for this study.
5. Mawn *et al.*, 1993.
6. Maeland *et al.*, 1997.
7. Maeland *et al.*, 2000.

Table 7. Specificity and expected lengths of amplicons of using different primer pairs.

Primer pairs*	Specificity	Length of amplicons (base pairs)	Protein profile code
IgAagGBS/ RIgAagGBS	*bac*	532-838	B
IgAS1/IgAA1	*bac*	303-591	B
GBS1360S/ GBS1937A	*bac*	652	B
GBS1717S/ GBS1937A	*bac*	292	B
bcaS1/bcaA	5'-end of *bca*	390	A
bcaS2/bcaA	5'-end of *bca*	342	A
BcaRUS/bcaRUA	*bca* repetitive unit/ *bca* repetitive unit–like region	235	a/as
bcaS1/balA	*alp2/alp3*	446	alp2 or alp3
bcaS2/balA	*alp2/alp3*	398	alp2 or alp3
balS/balA	*alp2/alp3*	302	alp2 or alp3
bal23S1/bal2A1	*alp2*	334	alp2
bal23S2/bal2A1	*alp2*	253	alp2
bal23S1/bal2A2	*alp2*	426	alp2
bal23S2/bal2A2	*alp2*	345	alp2
bal23S1/bal3A	*alp3*	321	alp3
bal23S2/bal3A	*alp3*	240	alp3
#ribS1/ribA3	*rib/rib-like*	355	R/r
ribS2/ribA1	*rib*	194	R
ribS2/ribA2	*rib*	225	R
ribS2/ribA3	*rib*	333	R

Notes.

*See Table 6 for primer sequences.

#For sequencing use only, not entirely specific for rib gene (see text for more detail).

Table 8. Genetic groups and subgroups of *bac* gene (C beta protein gene) based on amplicon length (using primers IgAagGBS/RIgAagGBS) and sequence heterogeneity.

Group or Subgroup	N=	Amplicon length	GenBank accession numbers	No. of different sites compared with (c.f.) main group	Molecular serotype/ serosubtypes
B1	19	532	X58470		17 = Ib; 2 = II
B1a	1	532	AF362686	1 (c.f. B1)	Ib
B2	3	550	AF362687		Ib, II, III-4
B3	2	586	AF362688		2=Ib
B3a	1	586	AF362689	4 (c.f. B3)	V
B3b	1	586	AF362690	21 (c.f. B3)	VI
B3c	1	586	AF362691	24 (c.f. B3)	Ib
B4	8	604	AF362692		4 = Ib; 4 = II
B4a	1	604	AF362693	1 (c.f. B4)	II
B4b	2	604	AF362694	2 (c.f. B4)	2 = Ib
B5	2	622	AF362695		Ia, VI
B5a	1	622	AF362696	2 (c.f. B5)	Ia
B6	1	640	AF362697		Ib
B7	1	658	AF362698		Ib
B7a	1	658	AF362699	34 (c.f. B7)	VI
B8	1	712	AF362700		Ib
B9	2	748	AF362701		2 = II
B9a	1	748	AF362702	13 (c.f. B9)	Ib
B10	2	820	AF362703		2 = Ib
B11	1	838	AF362704		Ib

Note.
*See Table 9 for further details of serotype/serosubtype relationships with protein antigens.

Table 9. The relationship between GBS protein gene profiles and capsular polysaccharide (cps) molecular serotypes/serosubtypes.

Serotype/ serosubtype *	N=	None	Aa	AaB	R	alp 3	a	as	alp2as	RB	R a
Ia	43	-	-	2	-	-	35	3	3	-	-
Ib	37	-	1	35	-	1	-	-	-	-	-
II	29	-	3	10	8	2	5	-	-	-	1
III-1	30	-	-	-	30	-	-	-	-	-	-
III-2	22	-	-	-	22	-	-	-	-	-	-
III-3	5	-	-	-	-	-	-	-	5	-	-
III-4	3	-	-	1	-	1	-	-	1	-	-
IV	9	-	-	-	1	-	8	-	-	-	-
V	38	1	-	-	1	35	-	-	-	1	-
VI	5	-	1	3	-	-	1	-	-	-	-
VII	1	-	-	-	-	1	-	-	-	-	-
VIII	2	1	-	-	-	1	-	-	-	-	-
Total	224	2	5	51	62	41	49	3	9	1	1

Note.

*See text for explanation of cps serosubtypes and Table 7 for explanation of protein antigen gene profile codes.

Table 10. Oligonucleotide primers used in this study.

Primer	Target	Tm°C[1]	GenBank accession numbers	Sequence[2]
IS861S	IS*861*	77.4	M22449	**445**GAG AAA ACA AGA GGG AGA CCG AGT AAA ATG GGA CG**479**
IS861A1	IS*861*	77.3	M22449	**831**CAC GAT TTC GCA GTT CTA AAT AAA TCC GAC GAT AGC C**795**
IS861A2	IS*861*	76.1	M22449	**1020**CAA ACT CCG TCA CAT CGG TAT AGC ACT TCT CAT AGG**985**
IS1548S	IS*1548*	76.5	Y14270	**143**CTA TTG ATG ATT GCG CAG TTG AAT TGG ATA GTC GTC**178**
IS1548S1	IS*1548*	77.0	Y14270	**539**GTT TGG GAC AGG TAG CGG TTG AGG AGA AAA GTA ATG**574**
IS1548A1	IS*1548*	77.0	Y14270	**574**CAT TAC TTT TCT CCT CAA CCG CTA CCT GTC CCA AAC**539**
IS1548A2	IS*1548*	70.3	Y14270	**915**CCC AAT ACC ACG TAA CTT ATG CCA TTT G**888**
IS1548A3	IS*1548*	78.0	Y14270	**930**CGT GTT ACG AGT CAT CCC AAT ACC ACG TAA CTT ATG CC**893**
IS1381S1	IS*1381*	80.1	AF064785/ AF367974	**272/818**CTT ATG AAC AAA TTG CGG CTG ATT TTG GCA TTC ACG**307/853**
IS1381S2	IS*1381*	81.7	AF064785/ AF367974	**497/1040**GGC TCA GGC GAT TGT CAC AAG CCA AGG GAG**526/1069**

IS1381A	IS*1381*	73.1	AF064785/ AF367974	**881/1424**CTA AAA TCC TAG TTC ACG GTT GAT CAT TCC AGC**849/1392**
ISSa4S	ISSa*4*	78.5	AF165983	**326**CGT ATC TGT CAC TTA TTT CCC TGC GGG TGT CTC C**359**
ISSa4A1	ISSa*4*	75.2	AF165983	**639**GCC GAT GTC ACA ACA TAG TTC AGG ATA TAG CCA G**606**
ISSa4A2	ISSa*4*	74.5	AF165983	**780**CGT AAA GGA GTC CAA AGA TGA TAG CCT TTT TGA ACC**745**
GBSi1S1	GBSi1	78.6	AJ292930	**721**CAT CTC GGA ACA ATA TGC TCG AAG CTT ACA AGC AAG TG**758**
GBSi1S2	GBSi1	77.3	AJ292930	**789**GGG GTC ACT ATC GAG CAG ATG GAT GAC TAT CTT CAC**824**
GBSi1A1	GBSi1	83.9	AJ292930	**1058**AAT GGC TGT TTC GCA GGA GCG ATT GGG TCT GAA CC**1024**
GBSi1A2	GBSi1	80.5	AJ292930	**1161**CCA GGG ACA TCA ATC TGT CTT GCG GAA CAG TAT CG**1127**

Notes.

1. The primer Tm values were provided by the primer synthesiser (Sigma-Aldrich).

2. Numbers represent the numbered base positions at which primer sequences start and finish (numbering start point "1" refers to the start point "1" of corresponding gene GenBank accession number).

Table 11. Specificity and expected lengths of amplicons of using different oligonucleotide primer pairs.

Primer pairs*	Specificity	Length of amplicons (base pairs)
IS861S/IS861A1	IS*861*	387
IS861S/IS861A2	IS*861*	576
IS1548S/IS1548A1	IS*1548*	432
IS1548S/IS1548A2	IS*1548*	773
IS1548S/IS1548A3	IS*1548*	788
IS1548S1/IS1548A2	IS*1548*	377
IS1548S1/IS1548A3	IS*1548*	392
IS1381S1/IS1381A	IS*1381*	610/607#
IS1381S2/IS1381A	IS*1381*	385
ISSa4S/ISSa4A1	ISSa*4*	314
ISSa4S/ISSa4A2	ISSa*4*	455
GBSi1S1/GBSi1A1	GBSi1	338
GBSi1S1/GBSi1A2	GBSi1	441
GBSi1S2/GBSi1A1	GBSi1	270
GBSi1S2/GBSi1A2	GBSi1	373

Notes.

*See table 10 for primer sequences.

Our sequencing result (GenBank accession number: AF367974) was 3 bp shorter than that previously described by Tamura et al., 2000 (GenBank accession number: AF064785).

Table 12. Relationship between mobile genetic elements and capsular polysaccharide serotypes, serotype III subtypes and surface protein gene profiles.

Serotype/ serosubtype	Protein gene profile	N=	IS*861*	IS*1548*	IS*1381*	ISSa *4*	GBSi1	No mobile element
Ia	AaB	2	2	-	2	-	-	-
Ia	alp2as	3	-	-	-	-	-	3
Ia	a	35	3	1	35	1	-	-
Ia	as	3	-	-	3	-	-	-
subtotal		*43*	*5*	*1*	*40*	*1*	-	*3*
Ib	Aa	1	-	-	-	-	-	1
Ib	AaB	35	30	-	35	1	-	-
Ib	alp3	1	-	-	1	-	-	-
subtotal		*37*	*30*	-	*36*	*1*	-	*1*
II	Aa	3	3	1	3	2	1	-
II	AaB	10	10	5	10	5	1	-
II	alp3	2	1	1	2	-	-	-
II	R	8	8	-	8	-	8	-
II	Ra	1	1	-	-	-	1	-
II	a	5	2	2	5	3	5	-
subtotal		*29*	*25*	*9*	*28*	*10*	*16*	-
III-1	R	30	30	30	30	1	-	-
III-2	R	22	22	-	-	-	22	-
III-3	alp2as	5	-	-	-	-	-	5
III-4	AaB	1	1	-	1	-	1	-
III-4	alp2as	1	-	-	-	-	1	-
III-4	alp3	1	-	-	1		1	-
subtotal		*60*	*53*	*30*	*32*	*1*	*25*	*5*
IV	R	1	1	-	1	-	1	-
IV	a	8	2	-	8	-	-	-
subtotal		*9*	*3*	-	*9*	-	*1*	-
V	alp3	35	3	1	35	1	1	-
V	R	1	1	-	1	1	-	-
V	RB	1	1	-	1	-	-	-
V	none	1	-	-	-	-	-	1
subtotal		*38*	*5*	*1*	*37*	*1*	*1*	*2*

VI	Aa	1	-	-	1	-	-	-
	AaB	3	3	-	3	-	-	-
	a	1	-	-	1	-	-	-
subtotal		*5*	*3*	-	*5*	-	-	-
VII	alp3	1	-	-	1	-	-	-
VIII	alp3	1	-	-	1	-	-	-
	none	1	-	-	1	-	-	-
subtotal		*2*	-	-	*2*	-	-	-
Total		*224*	*124 (55)*	*41 (18)*	*190 (85)*	*15 (7)*	*43 (19)*	*10 (4)*

Note.

A: 5'-end of *bca* gene (C alpha protein);

a: *bca* gene repetitive unit or *bca* gene repetitive unit-like sequence (multiple band amplicon);

as: *bca* gene repetitive unit or *bca* gene repetitive unit-like sequence (single band amplicon);

B: C beta/IgA binding protein (*bac*) gene.

R: Rib protein (*rib*) gene;

alp2: C alpha-like protein 2 (*alp2*) gene;

alp3: C alpha-like protein 3 (*alp3*) gene;

r: assumed Rib-like protein gene.

Table 13. Distribution of mobile genetic elements among 194 invasive GBS isolates.

	Mobile genetic elements present					
Total N =	IS*1381*	IS*861*	IS*1548*	IS*Sa4*	GBSi1	None
6	–	–	–	–	–	6
78	78	–	–	–	–	–
2	–	–	–	–	2	–
37	37	37	–	–	–	–
1	1	–	1	–	–	–
3	3	–	–	3	–	–
29	29	29	29	–	–	–
6	6	6	–	6	–	–
8	8	8	–	–	8	–
18	-	18	–	–	18	–
1	1	–	–	–	1	–
1	1	–	1	–	1	–
2	2	2	2	–	2	–
2	2	–	–	2	2	–
Total (n=194)	168 (87%)	100 (52%)	33 (17%)	11 (6%)	34 (18%)	6 (3%)

Note.

Data are numbers of isolates containing various combinations of mge

Table 14 Relationship between GBS genotypes and invasive disease age.

Serotype / Genotype	Age-group/disease[1]						
	0-6d	7-3m	4m –14yr	15-45 yr	46-60 yr	>60 yr	Total
Ia-1	14	4+1	1	7	3	6	35+1 (19%)
Ia-(2-8)	4	2	-	1	-	3	10
Ia total	18 (34%)	6+1 (21%)	1 (10%)	8 (28%)	3 (18%)	9 (17%)	45+1 (24%)
Ib-1	2	1+1	-	3	2	5+1	13+2
Ib-(2-16)	3	4+2	-	3	1	5	16+2
Ib total	5 (9.4%)	5+3 (24%)	-	6 (21%)	3	10+1	29+4 (17%)
II	8 (15%)	1 (3%)	-	4+1 (17%)	1	4 (7%)	18+1 (10%)
III-1	6+1 (13%)	4 (12%)	1+1 (20%)	1+1 (7%)	6+1 (41%)	4	22+4 (13%)
III-2	5 (9%)	5+4 (39%)[3]	1 (10%)	2	-	-	13+4 (9%)
III-(3-4)	1+1	1	-	1	1	1	5+1
III total	12+2 (26%)	10+4 (41%)	2+1 (30%)	4+1 (17%)	7+1 (44%)	5 (9%)	40+9 (25%)
IV total	3	-	-	-	-	4	7 (4%)
V-1	3	3	2	4	2	13+1	27+1 (14%)
V-(2-7)	1	1	-	1	-	4	7
V total	4 (8%)	4 (12%)	2 (20%)	5 (17%)	2 (11%)	17+1 (33%)[4]	34+1 (18%)
VI total	1	-	-	-	+1	3	4+1 (3%)
TOTAL	51+2=53	26+8=34	5+2=7	27+1=29	16+2=18	52+2=54	177+17=194

Notes:

1. Numbers after "+" refer to CSF isolates; all others are from blood.
2. Five aged 4m-1yr and one case was aged 3 yr.
3. Sst III-2 in late onset infection compared with all other groups: p=0.0005, odds ratio (OR) 6.8; 95% confidence interval (CI) 2.4-19.4.

MS-V in elderly compared with all other age-groups: p=0.001, OR 0.28; 95% CI 0.13-0.59).

CLAIMS

1. A method of typing a group B streptococcal bacterium which method comprises analysing the nucleotide sequence of one or more regions within the *cpsD, cpsE, cpsF, cpsG* and/or *cpsI/M* genes of said bacterium, said region(s) comprising one or more nucleotides whose sequence varies between types.

2. A method according to claim 1 wherein the nucleotide sequence is analysed for one or more positions corresponding to positions 62, 78-86, 138, 139, 144, 198, 204, 211, 281, 240, 249, 300, 321, 419, 429, 437, 457, 466, 486, 602, 606, 627, 636, 645, 803, 971, 1026, 1044, 1173, 1194, 1251, 1278, 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

3. A method according to claim 1 wherein at least one region is within a sequence delineated by the 3' 136 bases of the *cpsE* gene and the 5' 218 bases of the *cpsG* gene of the *cpsE-cpsF-cspG* gene cluster of said streptococcal bacterium.

4. A method according to claim 3 wherein the nucleotide sequence is analysed for one or more positions corresponding to positions 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

5. A method according to any one of claims 1 to 4 wherein at least one region is within the *cpsI/M* genes of said bacterium.

6. A method according to any one of claims 1 to 5 wherein the nucleotide sequence analysis step comprises sequencing said one or more regions.

7. A method according to any one of claims 1 to 5 wherein the nucleotide sequence analysis step comprises determining whether a polynucleotide obtained from said bacterium selectively hybridises to a polynucleotide probe comprising one or more of the said regions.

8. A method according to claim 7 which comprises determining whether the polynucleotide obtained from said bacterium hybridises to one or more of a plurality of polynucleotide probes corresponding to one or more of the said regions.

9. A method according to claim 9 wherein the plurality of polynucleotide probes are present as a microarray.

10. A method according to any one of claims 1 to 5 wherein the nucleotide sequence analysis step comprises an amplification step using one or more primers, at least one of which hybridises specifically to a sequence which differs between types.

11. A method according to any one of claims 1 to 6 wherein the nucleotide sequence analysis step comprises an amplification step using primer pairs, at least one of which hybridise specifically to a sequence which differs between types.

12. A method according to claim 10 or claim 11 wherein said primers are selected from the primers shown in Table 2.

13. A method of typing a group B streptococcal bacterium which method comprises determining the presence or absence in the genome of said bacterium of one or more surface protein genes selected from *rib*, *alp2* or *alp3* genes.

14. A method according to claim 13 wherein determining the presence or absence of said surface protein genes comprises determining whether a polynucleotide obtained from said bacterium selectively hybridises to a polynucleotide probe corresponding to a region of said surface protein genes.

15. A method according to any one of claim 13 wherein determining the presence or absence of said surface protein genes comprises an amplification step using one or more primers which amplify specifically a region of said surface protein genes.

16. A method according to claim 15 wherein said primers are selected from the primers shown in Table 6.

17. A method according to any one of claims 1 to 12 which further comprises determining the presence or absence of in the genome of said bacterium of one or more surface protein genes selected from *rib*, *alp2* or *alp3* genes.

18. A method of typing a group B streptococcal bacterium which method comprises determining the presence or absence in the genome of said bacterium of one or more mobile genetic elements selected from IS*861*, IS*1548*, IS*1381*, ISSa*4* and GBSi1.

19. A method according to claim 18 wherein determining the presence or absence of said mobile genetic elements comprises determining whether a polynucleotide obtained from said bacterium selectively hybridises to a polynucleotide probe corresponding to a region of said mobile genetic elements.

20. A method according to any one of claim 18 wherein determining the presence or absence of said mobile genetic elements comprises an amplification step using one or more primers which amplify specifically a region of said mobile genetic elements.

21. A method according to claim 20 wherein said primers are selected from the primers shown in Table 10.

22. A method according to any one of claims 13 to 17 which further comprises determining the presence or absence in the genome of said bacterium of one or more mobile genetic elements selected from IS*861*, IS*1548*, , IS*1381*, ISSa*4* and GBSi1.

23. A polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within a *cpsD-cpsE-cpsF-cpsG* gene of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between group B streptococcal serotypes.

24. A polynucleotide according to claim 23 wherein said nucleotides which differ between group B streptococcal serotypes correspond to one or more of positions 62, 78-86, 138, 139, 144, 198, 204, 211, 281, 240, 249, 300, 321, 419, 429, 437, 457, 466, 486, 602, 606, 627, 636, 645, 803, 971, 1026, 1044, 1173, 1194, 1251, 1278, 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

25. A polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within a sequence delineated by the 3' 136 base pairs of

cpsE and the 5' 218 base pairs of *cpsG* of the *cpsE-cpsF-cspG* gene cluster of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between group B streptococcal types.

26. A polynucleotide according to claim 25 wherein said nucleotides which differ between group B streptococcal types correspond to one or more of positions 1413, 1495, 1500, 1501, 1512, 1518, 1527, 1595, 1611, 1620, 1627, 1629, 1655, 1832, 1856, 1866, 1871, 1892, 1971, 2026, 2088, 2134, 2187 and 2196 as shown in Figure 1.

27. A polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within a *cpsI/M* gene of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between streptococcal serotypes.

28. A polynucleotide according to claim 27 wherein the polynucleotide is selected from the nucleotide sequences shown in Table 2.

29. A polynucleotide consisting essentially of at least 10 contiguous nucleotides corresponding to a region within a *rib, alp2* or *alp3* gene of a group B streptococcal bacterium, said polynucleotide comprising one or more nucleotides which differ between group B streptococcal subtypes.

30. A polynucleotide according to claim 29 wherein the polynucleotide is selected from the nucleotide sequences shown in Table 6.

31. Use of a polynucleotide according to any one of claims 23 to 30 in a method of serotyping and/or subtyping a group B streptococcal bacterium.

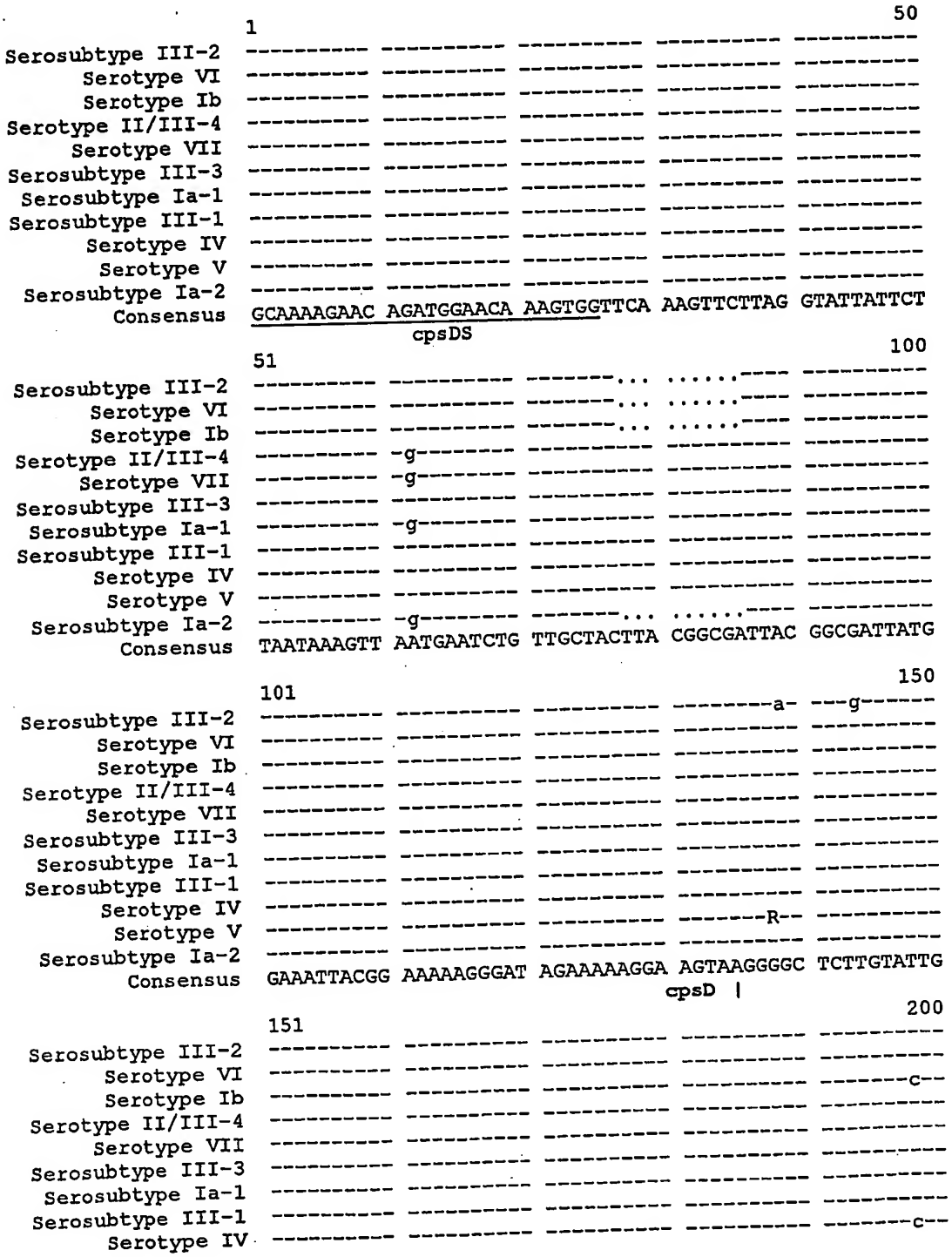
32. A composition comprising a plurality of polynucleotides according to any one of claims 23 to 30.

33. Use of a composition according to claim 32 in a method of serotyping and/or subtyping a group B streptococcal bacterium.

34. A microarray comprising a plurality of polynucleotides according to any one of claims 23 to 30.

35. Use of a microarray according to claim 34 in a method of serotyping and/or subtyping a group B streptococcal bacterium.

Figure 1. Multiple sequence alignments of the regions of the 3′ end of cpsD-cpsE-cpsF-and the 5′ end of cpsG for reference strains of serotypes Ia to VII.



```
       Serotype V ---------- ---------- ---------- ---------- -------M--
 Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
        Consensus AAAGAAAAAG AAAATATACA AAAGATTATT ATAGCGATGA TTCAAACAGT
                                                    | cpsE

                  201                                                250
Serosubtype III-2 ---a------ ---------- ---------- ---------- ----------
      Serotype VI ---------- g-----t--- ---------- ---------c ----------
      Serotype Ib ---------- ---------- ---------- ---------- --------c-
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
     Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---a------ ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- --------c-
       Serotype V ---------- ---------- ---------- ---------- --------Y-
 Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
        Consensus TGTGGTTTAT TTTTCTGCAA GTTTGACATT AACATTAATT ACTCCCAATT

                  251                                                300
Serosubtype III-2 ---------- ---------- ---------- ---------- ---------t
      Serotype VI ---------- ---------- ---------- ---------- ----------
      Serotype Ib ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
     Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- ----------
       Serotype V ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
        Consensus TTAAAAGCAA TAAAGATTTA TTGTTTGTTC TATTGATACA TTATATTGTC

                  301                                                350
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
      Serotype VI ---------- ---------- ---------- ---------- ----------
      Serotype Ib ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
     Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- t--------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- ----------
       Serotype V ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
        Consensus TTTTATCTTT CTGATTTTTA CAGAGACTTT TGGAGTCGTG GCTATCTTGA
                                              cpsES

                  351                                                400
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
      Serotype VI ---------- ---------- ---------- ---------- ----------
      Serotype Ib ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
     Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
```

```
                    ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
Consensus           AGAGTTTAAA ATGGTATTGA AATACAGCTT TTACTATATT TTCATATCAA

                    401                                                450
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----------
Serotype Ib         ---------- --------c- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- --------a- ---------- ----------
Serotype VII        ---------- ---------- --------a- ------t--- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-1    ---------- ---------- --------a- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- --------a- ---------- ----------
Consensus           GTTCATTATT TTTTATTTTT AAAAACTCTT TTACAACGAC ACGACTTTCC
                                                                 cpsEA1
                    451                                                500
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----------
Serotype Ib         ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ------c--- ---------- ---------- ---------- ----------
Serotype VII        ------c--- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- -----g---- ----------
Serosubtype Ia-1    ------t--- ---------- ---------- -----g---- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- -----a---- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ------t--- ---------- ---------- -----g---- ----------
Consensus           TTTTTTACTT TTATTGCTAT GAATTCGATT TTATTATATC TATTGAATTC

                    501                                                550
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----------
Serotype Ib         ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-1    ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
Consensus           ATTTTTAAAA TATTATCGAA AATATTCTTA CGCTAAGTTT TCACGAGATA

                    551                                                600
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----------
Serotype Ib         ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-1    ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
```

```
                Serotype V  ---------- ---------- ---------- ---------- ----------
          Serosubtype Ia-2  ---------- ---------- ---------- ---------- ----------
                 Consensus  CCAAAGTTGT TTTGATAACG AATAAGGATT CTTTATCAAA AATGACCTTT

                            601                                                650
          Serosubtype III-2  ---------- ---------- ---------- ---------- ----------
                Serotype VI  -----c---- ---------- ---------- ---------- ----------
                Serotype Ib  ---------- ---------- ---------- ---------- ----t-----
          Serotype II/III-4  -a-------- ---------- ---------- ---------- ----------
               Serotype VII  -a-------- ---------- ---------- ---------- ----------
          Serosubtype III-3  ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-1  ---------- ---------- ------t--- -----c---- ----------
          Serosubtype III-1  ---------- ---------- ---------- -----c---- ----------
                Serotype IV  ---------- ---------- ---------- ---------- ----t-----
                 Serotype V  ---------- ---------- ---------- ---------- ----t-----
           Serosubtype Ia-2  ---------- ---------- ------t--- -----c---- ----------
                 Consensus  AGGAATAAAT ACGACCATAA TTATATCGCT GTCTGTATCT TGGACTCCTC

                            651                                                700
          Serosubtype III-2  ---------- ---------- ---------- ---------- ----------
                Serotype VI  ---------- ---------- ---------- ---------- ----------
                Serotype Ib  ---------- ---------- ---------- ---------- ----------
          Serotype II/III-4  ---------- ---------- ---------- ---------- ----------
               Serotype VII  ---------- ---------- ---------- ---------- ----------
          Serosubtype III-3  ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-1  ---------- ---------- ---------- ---------- ----------
          Serosubtype III-1  ---------- ---------- ---------- ---------- ----------
                Serotype IV  ---------- ---------- ---------- ---------- ----------
                 Serotype V  ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-2  ---------- ---------- ---------- ---------- ----------
                 Consensus  TGAAAAGGAT TGTTATGATT TGAAACATAA CTCGTTAAGG ATAATAAACA
                            cpsES1
                            701                                                750
          Serosubtype III-2  ---------- ---------- ---------- ---------- ----------
                Serotype VI  ---------- ---------- ---------- ---------- ----------
                Serotype Ib  ---------- ---------- ---------- ---------- ----------
          Serotype II/III-4  ---------- ---------- ---------- ---------- ----------
               Serotype VII  ---------- ---------- ---------- ---------- ----------
          Serosubtype III-3  ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-1  ---------- ---------- ---------- ---------- ----------
          Serosubtype III-1  ---------- ---------- ---------- ---------- ----------
                Serotype IV  ---------- ---------- ---------- ---------- ----------
                 Serotype V  ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-2  ---------- ---------- ---------- ---------- ------K---
                 Consensus  AAGATGCTCT TACTTCAGAG TTAACCTGCT TAACTGTTGA TCAAGCTTTT
                                                                cpsEA2
                            751                                                800
          Serosubtype III-2  ---------- ---------- ---------- ---------- ----------
                Serotype VI  ---------- ---------- ---------- ---------- ----------
                Serotype Ib  ---------- ---------- ---------- ---------- ----------
          Serotype II/III-4  ---------- ---------- ---------- ---------- ----------
               Serotype VII  ---------- ---------- ---------- ---------- ----------
          Serosubtype III-3  ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-1  ---------- ---------- ---------- ---------- ----------
          Serosubtype III-1  ---------- ---------- ---------- ---------- ----------
                Serotype IV  ---------- ---------- ---------- ---------- ----------
                 Serotype V  ---------- ---------- ---------- ---------- ----------
```

```
Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
      Consensus  ATTAACATAC CCATTGAATT ATTTGGTAAA TACCAAATAC AAGATATTAT

                 801                                                850
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
     Serotype VI  --t------- ---------- ---------- ---------- ----------
     Serotype Ib  ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
    Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- ----------
       Serotype V ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
      Consensus  TAATGACATT GAAGCAATGG GAGTGATTGT CAATGTTAAT GTAGAGGCAC

                 851                                                900
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
     Serotype VI ---------- ---------- ---------- ---------- ----------
     Serotype Ib ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
    Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- ----------
       Serotype V ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
      Consensus  TTAGCTTTGA TAATATAGGA GAAAAGCGAA TCCAAACTTT TGAAGGATAT

                 901                                                950
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
     Serotype VI ---------- ---------- ---------- ---------- ----------
     Serotype Ib ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
    Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- ----------
       Serotype V ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
      Consensus  AGTGTTATTA CATATTCTAT GAAATTCTAT AAATATAGTC ACCTTATAGC

                 951                                               1000
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
     Serotype VI ---------- ---------- t--------- ---------- ----------
     Serotype Ib ---------- ---------- t--------- ---------- ----------
Serotype II/III-4 ---------- ---------- t--------- ---------- ----------
    Serotype VII ---------- ---------- t--------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
```

```
                    1001                                                1050
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
Consensus           AAAACGATTT TTGGATATCA CGGGTGCTAT TATAGGTTTG CTCATATGTG

                    1001                                                1050
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ---c------
Serotype Ib         ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- -----a---- ---------- ----------
Serosubtype Ia-1    ---------- ---------- -----a---- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- -----a---- ---------- ----------
Serotype V          ---------- ---------- -----a---- ---------- ----------
Serosubtype Ia-2    ---------- ---------- -----a---- ---------- ----------
Consensus           GCATTGTGGC AATTTTTCTA GTTCCGCAAA TCAGAAAAGA TGGTGGACCG

                    1051                                                1100
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----------
Serotype Ib         ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-1    ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
Consensus           GCTATCTTTT CTCAAAATAG AGTAGGTCGT AATGGTAGGA TTTTTAGATT
                    cpsES2
                    1101                                                1150
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----------
Serotype Ib         ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-1    ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
Consensus           CTATAAATTC AGATCAATGC GAGTAGATGC AGAACAAATT AAGAAAGATT
                                                  cpsEA3
                    1151                                                1200
Serosubtype III-2   ---------- ---------- ---------- ---------- ---a------
Serotype VI         ---------- ---------- ---------- ---------- ---a------
Serotype Ib         ---------- ---------- --g------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ---a------
Serosubtype Ia-1    ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ---a------
Serotype IV         ---------- ---------- ---------- ---------- ---a------
```

```
          Serotype V  ---------- ---------- ---------- ---------- ----------
   Serosubtype Ia-2  ---------- ---------- ---------- ---------- ----------
           Consensus  TATTAGTTCA CAATCAAATG ACAGGGCTAA TGTTTAAGTT AGACGATGAT

                      1201                                              1250
  Serosubtype III-2  ---------- ---------- ---------- ---------- ----------
         Serotype VI  ---------- ---------- ---------- ---------- ----------
         Serotype Ib  ---------- ---------- ---------- ---------- ----------
    Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
        Serotype VII  ---------- ---------- ---------- ---------- ----------
  Serosubtype III-3  ---------- ---------- ---------- ---------- ----------
    Serosubtype Ia-1  ---------- ---------- ---------- ---------- ----------
  Serosubtype III-1  ---------- ---------- ---------- ---------- ----------
         Serotype IV  ---------- ---------- ---------- ---------- ----------
          Serotype V  ---------- ---------- ---------- ---------- ----------
    Serosubtype Ia-2  ---------- ---------- ---------- ---------- ----------
           Consensus  CCTAGAATTA CTAAAATAGG AAAATTTATT CGAAAAACAA GCATAGATGA

                      1251                                              1300
  Serosubtype III-2  ---------- ---------- ---------- ---------- ----------
         Serotype VI  a--------- ---------- ---------- ---------- ----------
         Serotype Ib  ---------- ---------- ---------- ---------- ----------
    Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
        Serotype VII  ---------- ---------- ---------- ---------- ----------
  Serosubtype III-3  ---------- ---------- ---------- ---------- ----------
    Serosubtype Ia-1  ---------- ---------- ---------- ---------- ----------
  Serosubtype III-1  ---------- ---------- ---------- ---------- ----------
         Serotype IV  ---------- ---------- ---------- ---------- ----------
          Serotype V  ---------- ---------- -------g-- ---------- ----------
    Serosubtype Ia-2  ---------- ---------- ---------- ---------- ----------
           Consensus  GTTGCCTCAA TTCTATAATG TTTTAAAAGG TGATATGAGT TTAGTAGGAA

                      1301                                              1350
  Serosubtype III-2  ---------- ---------- ---------- ---------- ----------
         Serotype VI  ---------- ---------- ---------- ---------- ----------
         Serotype Ib  ---------- ---------- ---------- ---------- ----------
    Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
        Serotype VII  ---------- ---------- ---------- ---------- ----------
  Serosubtype III-3  ---------- ---------- ---------- ---------- ----------
    Serosubtype Ia-1  ---------- ---------- ---------- ---------- ----------
  Serosubtype III-1  ---------- ---------- ---------- ---------- ----------
         Serotype IV  ---------- ---------- ---------- ---------- ----------
          Serotype V  ---------- ---------- ---------- ---------- ----------
    Serosubtype Ia-2  ---------- ---------- ---------- ---------- ----------
           Consensus  CACGCCCTCC CACAGTTGAT GAATATGAAA AGTATAATTC AACGCAGAAG

                      1351                                              1400
  Serosubtype III-2  ---------- ---------- ---------- ---------- ----------
         Serotype VI  ---------- ---------- ---------- ---------- ----------
         Serotype Ib  ---------- ---------- ---------- ---------- ----------
    Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
        Serotype VII  ---------- ---------- ---------- ---------- ----------
  Serosubtype III-3  ---------- ---------- ---------- ---------- ----------
    Serosubtype Ia-1  ---------- ---------- ---------- ---------- ----------
  Serosubtype III-1  ---------- ---------- ---------- ---------- ----------
```

```
                    1401                                                1450
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
Consensus           CGACGCCTTA GTTTTAAGCC AGGAATCACT GGTTTGTGGC AAATATCTGG

                    1401                                                1450
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----------
Serotype Ib         ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ----------c---------- ---------- ---------- ----------
Serosubtype Ia-1    ----------c---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ----------c---------- ---------- ---------- ----------
Consensus           TAGAAATAAT ATTACTGATT TTGATGAAAT CGTAAAGTTA GATGTTCAAT

                    1451                                                1500
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----a-----
Serotype Ib         ---------- ---------- ---------- ---------- ---------g
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-1    ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
Serotype V          ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
Consensus           ATATCAATGA ATGGTCTATT TGGTCAGATA TTAAGATTAT TCTCCTAACA
                          cpsES3
                    1501                                                1550
Serosubtype III-2   ----------t-----c-- ---------- ---------- ----------
Serotype VI         ----------t-----c-- ---------- ---------- ----------
Serotype Ib         ----------t-----c-- ---------- ---------- ----------
Serotype II/III-4   t--------- ---------- ---------- ---------- ----------
Serotype VII        t--------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ------t--- ---------- ----------
Serosubtype Ia-1    ---------- ---------- ------t--- ---------- ----------
Serosubtype III-1   ----------t-----c-- ---------- ---------- ----------
Serotype IV         ---------- ---------- ------t--- ---------- ----------
Serotype V          ----------t-----c-- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ------t--- ---------- ----------
Consensus           CTAAAGGTAG TCTTACTTGG GACAGGAGCT AAGTAAAGGT AAGGTTTGAA
                                                    cpsE  |   cpsEFA
                    1551                                                1600
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
Serotype VI         ---------- ---------- ---------- ---------- ----c-----
Serotype Ib         ---------- ---------- ---------- ---------- ----c-----
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
Serotype VII        ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-1    ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
Serotype IV         ---------- ---------- ---------- ---------- ----------
```

```
                Serotype V  ---------- ---------- ---------- ---------- ----------
          Serosubtype Ia-2  ---------- ---------- ---------- ---------- ----------
                Consensus   AGGAATATAA TGAAAATTTG TCTGGTTGGT TCAAGTGGTG GTCATCTAGC
                                      |  cpsF
                                1601                                          1650
          Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
                Serotype VI   ---------- ---------- ---------- ---------- ----------
                Serotype Ib   ---------- ---------- ---------- ---------- ----------
          Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
               Serotype VII   ---------- t--------t ---------- ---------- ----------
          Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-1   ---------- ---------- ---------- ---------- ----------
          Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
                Serotype IV   ---------- ---------- ------t--- ---------- ----------
                 Serotype V   ---------- ---------- ---------- ---------- ----------
          Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
                Consensus     ACACTTGAAC CTTTTGAAAC CCATTTGGGA AAAAGAAGAT AGGTTTTGGG

                                1651                                          1700
          Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
                Serotype VI   ---------- ---------- ---------- ---------- ----------
                Serotype Ib   ----t----- ---------- ---------- ---------- ----------
          Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
               Serotype VII   ---------- ---------- ---------- ---------- ----------
          Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-1   ---------- ---------- ---------- ---------- ----------
          Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
                Serotype IV   ---------- ---------- ---------- ---------- ----------
                 Serotype V   ---------- ---------- ---------- ---------- ----------
          Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
                Consensus     TAACCTTTGA TAAAGAAGAT GCTAGGAGTA TTCTAAGAGA AGAGATTGTA

                                1701                                          1750
          Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
                Serotype VI   ---------- ---------- ---------- ---------- ----------
                Serotype Ib   ---------- ---------- ---------- ---------- ----------
          Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
               Serotype VII   ---------- ---------- ---------- ---------- ----------
          Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-1   ---------- ---------- ---------- ---------- ----------
          Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
                Serotype IV   ---------- ---------- ---------- ---------- ----------
                 Serotype V   ---------- ---------- ---------- ---------- ----------
          Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
                Consensus     TATCATTGCT TCTTTCCAAC AAACCGTAAT GTCAAAAACT TGGTAAAAAA

                                1751                                          1800
          Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
                Serotype VI   ---------- ---------- ---------- ---------- ----------
                Serotype Ib   ---------- ---------- ---------- ---------- ----------
          Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
               Serotype VII   ---------- ---------- ---------- ---------- ----------
          Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
           Serosubtype Ia-1   ---------- ---------- ---------- ---------- ----------
```

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Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- ----------
       Serotype V ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
       Consensus  TACTATTCTA GCTTTTAAGG TCCTTAGAAA AGAAAGACCA GATGTTATCA

                  1801                                              1850
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
      Serotype VI ---------- ---------- ---------- ---------- ----------
      Serotype Ib ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
     Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- -t-------- ----------
       Serotype V ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
       Consensus  TATCATCTGG TGCCGCTGTA GCAGTACCAT TCTTTTATAT TGGTAAGTTA
                                    cpsFS
                  1851                                              1900
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
      Serotype VI ---------- ---------- ---------- ---------- ----------
      Serotype Ib -----c---- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
     Serotype VII ---------- -----a---- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- ----------
       Serotype V ---------- ---------- c--------- ---------- -g--------
 Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
       Consensus  TTTGGTTGTA AGACCGTTTA TATAGAGGTT TTCGACAGGA TAGATAAACC
                                    cpsFA
                  1901                                              1950
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
      Serotype VI ---------- ---------- ---------- ---------- ----------
      Serotype Ib ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
     Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
      Serotype IV ---------- ---------- ---------- ---------- ----------
       Serotype V ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-2 ---------- ---------- ---------- ---------- ----------
       Consensus  AACTTTGACA GGAAAATTAG TGTATCCTGT AACAGATAAA TTTATTGTTC

                  1951                                              2000
Serosubtype III-2 ---------- ---------- ---------- ---------- ----------
      Serotype VI ---------- ---------- a--------- ---------- ----------
      Serotype Ib ---------- ---------- ---------- ---------- ----------
Serotype II/III-4 ---------- ---------- ---------- ---------- ----------
     Serotype VII ---------- ---------- ---------- ---------- ----------
Serosubtype III-3 ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1 ---------- ---------- ---------- ---------- ----------
Serosubtype III-1 ---------- ---------- ---------- ---------- ----------
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                    ---------- ---------- ---------- ---------- ----------
      Serotype IV   ---------- ---------- ---------- ---------- ----------
      Serotype V    ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
        Consensus   AGTGGGAAGA AATGAAAAAA GTTTATCCTA AGGCAATTAA TTTAGGAGGA

                    2001                                              2050
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
      Serotype VI   ---------- ---------- ---------- ---------- ----------
      Serotype Ib   ---------- ---------- -----a---- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
     Serotype VII   ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1   ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
      Serotype IV   ---------- ---------- ---------- ---------- ----------
      Serotype V    ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
        Consensus   ATTTTTTAAT GATTTTTGTC ACAGTGGGGA CACATGAACA GCAGTTCAAC
                      cpsF  |  cpsG

                    2051                                              2100
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
      Serotype VI   ---------- ---------- ---------- ---------- ----------
      Serotype Ib   ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
     Serotype VII   ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1   ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- -------a-- ----------
      Serotype IV   ---------- ---------- ---------- ---------- ----------
      Serotype V    ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
        Consensus   CGTCTTATTA AAGAAGTTGA TAGATTAAAA GGGACAGGTG CTATTGATCA

                    2101                                              2150
Serosubtype III-2   ---------- ---------- ---------- ---c------ ----------
      Serotype VI   ---------- ---------- ---------- ---------- ----------
      Serotype Ib   ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ---------- ----------
     Serotype VII   ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1   ---------- ---------- ---------- ---c------ ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
      Serotype IV   ---------- ---------- ---------- ---------- ----------
      Serotype V    ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2    ---------- ---------- ---------- ---------- ----------
        Consensus   AGAAGTGTTC ATTCAAACGG GTTACTCAGA CTTTGAACCT CAGAATTGTC
                                                      cpsGS
                    2151                                              2200
Serosubtype III-2   ---------- ---------- ---------- ---------- ----------
      Serotype VI   ---------- ---------- ---------- ---------- ----------
      Serotype Ib   ---------- ---------- ---------- ---------- ----------
Serotype II/III-4   ---------- ---------- ---------- ------g--- -----g----
     Serotype VII   ---------- ---------- ---------- ---------- ----------
Serosubtype III-3   ---------- ---------- ---------- ---------- ----------
 Serosubtype Ia-1   ---------- ---------- ---------- ---------- ----------
Serosubtype III-1   ---------- ---------- ---------- ---------- ----------
```

```
      Serotype IV  ---------- ---------- ---------- ---------- ----------
       Serotype V  ---------- ---------- ---------- ---------- ----------
Serosubtype Ia-2   ---------- ---------- ---------- ---------- ----------
         Consensus AGTGGTCAAA ATTTCTCTCA TATGATGATA TGAACTCTTA CATGAAAGAA
                                                cpsGA1     cpsGA2

                           2201                    2226
Serosubtype III-2  ---------- ---------- ------
      Serotype VI  ---------- ---------- ------
      Serotype Ib  ---------- ---------- c-----
Serotype II/III-4  ---------- ---------- ------
     Serotype VII  ---------- ---------- ------
Serosubtype III-3  ---------- ---------- ------
 Serosubtype Ia-1  ---------- ---------- ------
Serosubtype III-1  ---------- ---------- ------
      Serotype IV  ---------- ---------- ------
       Serotype V  ---------- ---------- ------
Serosubtype Ia-2   ---------- ---------- ------
         Consensus GCTGAGATTG TTATCACACA TGGCGG
                   cpsGA3
```

Notes.
Numbering start point "1" refers to the start point "1" of GenBank accession number AF332908 (for serotype IV reference strain 3139).
Serosubtype Ia-1: strain 090, GenBank accession number AF332893;
Serosubtype Ia-2: strain NZRM 908(NCDC SS615), GenBank accession number AF332894;
Serotype Ib: strain H36B, GenBank accession number AF332903;
Serotype II/III-4: strain 18RS21, GenBank accession number AF332905;
Serosubtype III-1: strain SG99/056, GenBank accession number AF332899;
Serosubtype III-2: strain M781, GenBank accession number AF332896;
Serosubtype III-3: strain NZRM 912 (NCDC SS620), GenBank accession number AF332897;
III-4 (Subtype III-4): strain SG96/220, GenBank accession number AF363036;
Serotype IV: strain 3139, GenBank accession number AF332908;
Serotype V: strain CJB 111, GenBank accession number AF332910;
Serotype VI: strain SS1214, GenBank accession number AF332901;
Serotype VII: strain 7271, GenBank accession number AF332913.

Figure 2. Algorithm for GBS molecular serotype (MS) identification by PCR and sequencing.

A. GBS identification primer pairs.

Amplification primers	**Sag59/Sag190**	**or**	**DSF2/DSR1**
Result (band size)	**196bp**		**254bp**
Interpretation	**GBS positive**		

B. GBS MS identification.

1) MS-specific PCR.

Primers	**IacpsHS1/cpsIA**	**IbcpsIS/IbcpsIA1**	**IIIcpsHS/cpsIA**	**IVcpsHS1/IVcpsMA**	**VcpsHS2/VcpsMA**	**VIcpsHS/VIcpsHA1**
Result (band size)	**354bp**	**523bp**	**641bp**	**379bp**	**374bp**	**327bp**
Interpretation	**serotype Ia**	**serotype Ib**	**serotype III**	**serotype IV**	**serotype V**	**serotype VI**

2) Sequencing.

Amplification primers	**cpsES3/cpsGA1**	**(or cpsES3/cpsFA + cpsFS/cpsGA1)**		
Result (band size)	**790bp**	**(450bp**	**+**	**423bp)**
Sequencing primers	**cpsGA**	**(or cpsFA + cpsGA)**		
Interpretation	**identification serotypes according to the sequence heterogeneity**			

Figure 3. Multiple sequence alignments of the gene sequences of the cpsG-cpsH-cpsI/M for serotypes Ia, Ib, II, III, IV, V and VI (start and stop codons were highlighted).

```
                 1                                                   50
Serotype IV      ATGATTTTTG TCACAGTGGG GACACATGAA CAGCAGTTCA ACCGTCTTAT
 Serotype V      ATGATTTTTG TCACAGTGGG GACACATGAA CAGCAGTTCA ACCGTCTTAT
Serotype Ia      ATGATTTTTG TCACAGTGGG GACACATGAA CAGCAGTTCA ACCGTCTTAT
Serotype Ib      ATGATTTTTG TCACAGTAGG GACACATGAA CAGCAGTTCA ACCGTCTTAT
Serotype III     ATGATTTTTG TCACAGTGGG GACACATGAA CAGCAGTTCA ACCGTCTTAT
Serotype VI      ATGATTTTTG TCACAGTGGG GACACATGAA CAGCAGTTCA ACCGTCTTAT
  Consensus      ********** *******_** ********** ********** **********
                 cpsG
                 51                                                  100
Serotype IV      TAAAGAAGTT GATAGATTAA AAGGGACAGA TGCTATTGAT CAAGAAGTGT
 Serotype V      TAAAGAAGTT GATAGATTAA AAGGGACAGG TGCTATTGAT CAAGAAGTGT
Serotype Ia      TAAAGAAGTT GATAGATTAA AAGGGACAGG TGCTATTGAT CAAGAAGTGT
Serotype Ib      TAAAGAAGTT GATAGATTAA AAGGGACAGG TGCTATTGAT CAAGAAGTGT
Serotype III     TAAAGAAGTT GATAGATTAA AAGGGACAGG TGCTATTGAT CAAGAAGTGT
Serotype VI      TAAAGAAGTT GATAGATTAA AAGGGACAGG TGCTATTGAT CAAGAAGTGT
  Consensus      ********** ********** *********_ ********** **********

                 101                                                 150
Serotype IV      TCATTCAAAC GGGTTACTCA GACTTTGAAC CTCAGAATTG TCAGTGGTCA
 Serotype V      TCATTCAAAC GGGTTACTCA GACTTTGAAC CTCAGAATTG TCAGTGGTCA
Serotype Ia      TCATTCAAAC GGGTTACTCA GACTTTGAAC CTCAGAATTG TCAGTGGTCA
Serotype Ib      TCATTCAAAC GGGTTACTCA GACTTTGAAC CTCAGAATTG TCAGTGGTCA
Serotype III     TCATTCAAAC GGGTTACTCA GACTTCGAAC CTCAGAATTG TCAGTGGTCA
Serotype VI      TCATTCAAAC GGGTTACTCA GACTTTGAAC CTCAGAATTG TCAGTGGTCA
  Consensus      ********** ********** *****_**** ********** **********

                 151                                                 200
Serotype IV      AAATTTCTCT CATATGATGA TATGAACTCT TACATGAAAG AAGCTGAGAT
 Serotype V      AAATTTCTCT CATATGATGA TATGAACTCT TACATGAAAG AAGCTGAGAT
Serotype Ia      AAATTTCTCT CATATGATGA TATGAACTCT TACATGAAAG AAGCTGAGAT
Serotype Ib      AAATTTCTCT CATATGATGA TATGAACTCT TACATGAAAG AAGCTGAGAT
Serotype III     AAATTTCTCT CATATGATGA TATGAACTCT TACATGAAAG AAGCTGAGAT
Serotype VI      AAATTTCTCT CATATGATGA TATGAACTCT TACATGAAAG AAGCTGAGAT
  Consensus      ********** ********** ********** ********** **********

                 201                                                 250
Serotype IV      TGTTATCACA CATGGCGGTC CAGCGACGTT TATGAATGCA GTTTCTAAAG
 Serotype V      TGTTATCACA CATGGCGGCC CAGCGACGTT TATGAATGCA GTTTCTAAAG
Serotype Ia      TGTTATCACA CATGGCGGTC CAGCGACGTT TATGAATGCA GTTTCTAAAG
Serotype Ib      TGTTATCACA CACGGCGGTC CAGCAACGTT TATGAATGCA GTTTCTAAAG
Serotype III     TGTTATCACA CATGGCGGCC CAGCGACGTT TATGTCAGTT ATTTCTTTAG
Serotype VI      TGTTATCACA CATGGCGGCC CAGCGACGTT TATGTCAGTT ATTTCTTTAG
  Consensus      ********** **_******_* ****_***** ****___*__ _******__**
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               251                                                300
Serotype IV    GGAAAAAAAC TATTGTGGTT CCTAGACAAG AACAGTTTGG AGAGCATGTG
Serotype V     GAAAAAAAAC TATTGTGGTT CCTAGACAAG AACAGTTTGG AGAGCATGTG
Serotype Ia    GGAAAAAAAC TATTGTGGTT CCTAGACAAG AACAGTTTGG AGAGCATGTG
Serotype Ib    GGAAAAAAAC TATTGTGGTT CCTAGACAAG AACAGTTTGG AGAGCATGTG
Serotype III   GGAAATTACC AGTTGTTGTT CCTAGGAGAA AGCAGTTTGG TGAACATATC
Serotype VI    GGAAATTACC AGTTGTTGTT CCCAGGAGAA AGCAGTTTGG TGAACATATC
Consensus      *-***--*-* --****-*** **-**---*- *-******** -**-***-*-

               301                                                350
Serotype IV    AATAATCATC AGGTGGATTT TGTTAATAAG GTAAAAACAA TGTATAATTT
Serotype V     AATAATCATC AGGTGGACTT TGTTAATAAG GTAAAAACAA TGTATAATTT
Serotype Ia    AATAATCATC AGGTGGATTT TTTGAAAGAG TTATTCTTGA AAATTGAATT
Serotype Ib    AATAATCATC AGGTGGATTT TTTGAAAGAG TTATTCTTGA AATATGAGTT
Serotype III   AATGATCATC AAATACAATT TTTAAAAAAA ATTGCCCACC TGTATCCCTT
Serotype VI    AATGATCATC AAATACAATT TTTAAATTCG ATTGCCCACC TGTATCCCTT
Consensus      ***-****** *--*--*-** *-*-**---- -*-------- ----*---**

               351                                                400
Serotype IV    TGATATCGTT GTAGATATTG AAAGGTTACA AAATGTAGTC TATGAGGGGA
Serotype V     TGATATCGTT GTAGATATTG AAAGGTTACA AAATGTAGTC TATGAGGGAA
Serotype Ia    AGATTATATT TTGAATATCA GTGAATTAGA GAATATTATT AAGGAAAAAA
Serotype Ib    AGATTATATT TTGAATATCA GTGAATTAGA GAATATTATT AAGGAAAAAA
Serotype III   GGCTTGGATT GAAGATGTAG ATGGACTTGC GGAAGCGTT. ..GAAAAGGA
Serotype VI    GGCTTGGATT GAAGATGTAG ATGGACTTGC GGAAGCGTT. ..GAAAAGGA
Consensus      -*-*----** ----**-*-- ------*--- --*-----*- ----*----*

               401                                                450
Serotype IV    CGATGAATCG TCCGTTTTTA GAAACTAACA GAAGTAATTT TATT......
Serotype V     TGATGAATCG TCCGTTTTTA GAAACTAATA GTAGTAATTT TATT......
Serotype Ia    ATATATCTAC TAGTAAAGTA ATATCACAAA ACAATGATTT TTGTTTCTCT
Serotype Ib    ATATATCTAC TAGTAAAGTA ATATCACAAA ACAATGATTT TTGTTCCTCT
Serotype III   ATATAGCTAC AGAAAAATAT CAGGGAAATA ATGATATGTT TTGT......
Serotype VI    ATATAGCTAC AGAAAAATAT CAGGGAAATA ATGATATGTT TTGT......
Consensus      --**---*-- ---------- -------*-* ----*---** *--*------

               451                                                500
Serotype IV    .......... .......... ....GAAGAA TTTAAGGTAA TATTAAAGGA
Serotype V     .......... .......... ....GAAGAA TTTAAGGTAA TATTAAAGGA
Serotype Ia    TTCAAAAATG AACATTTCAT AAACTATTTG AATAAATATA TTTTGTTGGA
Serotype Ib    TTCAAAAATG AAC..TTTCT AAACTATTTG AATAAATATA TTTTGTTGGA
Serotype III   .......... .......... ......CATA AATTAGAAAA AATTATAGGT
Serotype VI    .......... .......... ......CATA AATTAGAAAA AATTATAGGT
Consensus      ---------- ---------- ---------- --*-*----* --**---**-

               501                                                550
Serotype IV    GTTGTGTGAT GAAA...... .ATCAATAAA AACTCTTTAT TTTATATTGC
Serotype V     GTTGTGCGAT GAAA...... .ATCAATAAA AACTCTTTAT TTTATATTGC
Serotype Ia    GAAAAAAATT GAAATTAACA TATCAATCCA AAGTATTTGT TAATAGGAGG
Serotype Ib    GAAAAAAATT GAAATTAAC. TATCAATCCA AAGTATTTGT TAATAGGAGG
Serotype III   GAAATATGAG GAAAT....A TCTAGATTTA GATTATTCTT TATTTTATGC
Serotype VI    GAAATATGAG GAAAT....A TCTAGATTTA GATTATTCTT TATTTTATGC
Consensus      *--------- ****------ --*--**--* -*-*-**--* *-------*-
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                551                                                       600
Serotype IV     AATATTTTTA GTTAATTTTT TTAAATCACT AGGTTTAGGA GAGGGGAACT
 Serotype V     AATATTTTTA GTTAATTTTT TTAAATCACT GGGTTTAGGC GAGGGAAACT
Serotype Ia     AATTTTCGCT TTAACCCTAT TTTCAAAGCC AATGCAACTT TTGTTACTTT
Serotype Ib     AATTTTCGCT TTAACCCTAT TTTCAAAGCC AATGCAACTT TTGTTACTTT
Serotype III    TCTTTGGGTA CTTATTTTAG TACCAAACCA ATGGTATCAG TTTTTAATTA
Serotype VI     TCTTTGGGTA CTTATTTTAG TACCAAACCA ATGGTATCAG TTTTTAATTA
  Consensus     --*-*----- -*-*---*-- *---*---*- ---------- ----------
                                                  cpsH
                601                                                       650
Serotype IV     CAACTTACAA AATAGTGATG TTTGTTGCAA TCTTCTTGTG TGGAATAAAA
 Serotype V     CAGCTTACAA AATAGTGATG TTAGTTGCAA TTTTACTGTG TGGAATAAAA
Serotype Ia     TAGCATTAAT AGTTTTACTT ATTTGTAGTA GTTATAAGAA AAAAATGAAA
Serotype Ib     TAGCATTAAT AGTTTTACTT ATTTGTAGTA GTTATAATGA AAAAATGAAA
Serotype III    TTACCATTAT AGTTCTATTA TTACTTTGGA AGAGTGAGTT TAGAAT...A
Serotype VI     TTACCATTAT AGTTCTATTA TTACTTTGGA AGAGTGAGTT TAGAAT...A
  Consensus     ---*----*- *-*--*--*- -*---*---* ---------- ---***---*

                651                                                       700
Serotype IV     TTTTTA.... ..TTAGATAG CCTTTATTTT GAAAGAAGAA AACTCGTTAT
 Serotype V     TTTTTA.... ..TTAGATAG CCTTTATTTT GAAAGAAGAA AACTCGTGAT
Serotype Ia     TTTTTATATA TGGCTGAAAT TTTTTTCATT GTATTTTATA TCATTTATTT
Serotype Ib     TTTTTAAATA TGGCTGAAAT TTTTTTCATT GTATTTTATA TGGTTTATTT
Serotype III    TCTATAAGCA ATTCTTCAAT ACTATTTCTG CTTTGGTTAT TTATTTATTT
Serotype VI     TCTATAAGCA ATTCTTCAAT ACTATTTCTG CTTTGGTTAT TTATTTATTT
  Consensus     *-*-**---- --------*- --*-*---*- ---------- ---*-----*

                701                                                       750
Serotype IV     CATCTTTTTA TTATTTATTG CGACCATTTT GAATTTATTC TTTGTTCATA
 Serotype V     CATCTTTTTA TTATTTATCG CGACCATTTT GAATTTATTC TTTGTTCATA
Serotype Ia     AACTTCAATA TTGCTACATT CTTTGTTTAA AACTCCTGAT TTTGATAGAA
Serotype Ib     AGTATCAATA GTATTAAATT CGTTATTTAG AAGTCCAGAA TTTCATAGAG
Serotype III    ATTTGCAATA CTCATTAGAG GTACTCAAGA GGATATAACG TTTCAGCGAT
Serotype VI     ATTTGCAATA CTCATTAGAG GTACTCAAGA GGATATAACG TTTCAGCGAT
  Consensus     ---------** -*--*----- ---------- ---*------ ***-------

                751                                                       800
Serotype IV     AGGTTACTTT TATATTAA.. .......... .........C TTTAATTTTT
 Serotype V     AGGTTACTTT TATATTAA.. .......... .........C TTTAATTTTT
Serotype Ia     TTTTAGCAGC TTTTAACTCG TTGATTATCG GTATAGTATC AGTGGCTTTG
Serotype Ib     TCATTGCTGC ATTCAATTCA CTGGCAGTAG GGGTTGTGTC CTTATTATTT
Serotype III    TTATTGCTGA GCTATTAAAA CTAATTAGTA CAGGATATGC TTTATTTTTT
Serotype VI     TTATTGCTGA GCTATTAAAA CTAATTAGTA CAGGATATGC TTTATTTTTT
  Consensus     ---*--*--- --*------- ---------- ---------* --*----**-

                801                                                       850
Serotype IV     TTTCTAGCAT TAAAGGATAT CTCTCTAAAA AAAGCTTTCT CTATAATAAT
 Serotype V     TTTCTAGCAT TAAAGGACAT CTCTCTAAAA AAAGCTTTCT CTATAATAAT
Serotype Ia     AAACGGTGGT ATAAGAATAC AACTTTGGAG TTAGATAAAA TATTAAAAGC
Serotype Ib     TACCATTACT ATAAGAATAC TAATATTGAA TTAACAAAAT TGCTAAAATC
Serotype III    TATAATTATT ATAGAAAAGC TGATTTTAAT AGTTCAGTTG TAAGGAATGT
Serotype VI     TATAATTATT ATAGAAAAGC TGATTTTAAT AGTTCAGTTG TAAGGAATGT
  Consensus     ----------* --*---*--- ---*-*--*- ---------- -----*----
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                851                                                       900
Serotype IV     AGGATCGCGT ATTTTGGGAG TTCTATTAAA TCAAATTTTT GTGAAATTAG
 Serotype V     AGGATCGCGT ATTTTGGGAG TTCTATTAAA TCAAATTTTT GTGAAATTAG
Serotype Ia     ATTTTTATTT AATGGGTTAA TCCTATTTTT TTTAGGGGGA ACATATTATT
Serotype Ib     ATTTTTGTTT AATGCAATTA TTTTGTTTTG TTTAGGATTT CTATATTATT
Serotype III    GGTAAAGGTT AACTATTTTG TGTTGTTTCT TATAACAGTT TTATATT...
Serotype VI     GGTAAAGGTT AACTATTTTG TGTTGTTTCT TATAACAGTT TTATATT...
  Consensus     ---------* *--------- *--*-**--- *--*------ ----*-*---

                901                                                       950
Serotype IV     ATTTAATAGA AATTAAATAT ATCAATTTTT ATAGGGATGG ACAATTTATT
 Serotype V     ATTTAATAGA AATTAAGTAT GTCAATTTTT ATAGGGATGG ACAATTTATT
Serotype Ia     ATTGTTTGCA TAATAATATT CAAAATATCA GTATTTTTGG TAGAGATTTG
Serotype Ib     ATGCCATATA TTTTGATGTA GAGAATGTAA GTCTTTTTGG AAGAAATTTA
Serotype III    ......TATT TTTTCCTATG CTGAAGCCAA CTTTATTTGG AAGAGAATTG
Serotype VI     ......TATT TTTTCCAAAT GAATTTACTA CATTCCTAGG AAGAGATTTA
  Consensus     ------*--- ---*------ ---------- ---------* ---*----*-

                951                                                      1000
Serotype IV     CTGAGAAGTG ACT..... .......... .......... ......TAGG
 Serotype V     CTGAGAAGTG ACT..... .......... .......... ......TAGG
Serotype Ia     ATTGGGTCAG ACTGGATTAA TGGTATGCAT ACTCAAAGAG CAATGGGATT
Serotype Ib     ATTGGATCAG ATTGGATAAA TGGGATGCAT ACGCAGAGAG CAATGGCTTT
Serotype III    TTTTCAATAG AGTGGTTTCC ACATATG... AGAATAAGAC TTGCGGCATA
Serotype VI     TTTTCAATTG AATGGATTCC TTCTATG... AAAGTTAGAC TTACTGCATA
  Consensus     -*-------* *-*------- ---------- ---------- ----------

                1001                                                     1050
Serotype IV     TTTTGGTCAT CCTAACTTTA TTCATAATTT TTTTGCAGTA ACTGTTTTTT
 Serotype V     TTTTGGTCAT CCTAACTTTA TTCATAATTT TTTTGCTCTA ACTATTTTCT
Serotype Ia     TTTTGAATAT TCAAACCTTA TAATTCCTAT GACAGTGGTA ACTAACTATA
Serotype Ib     CTTTGAATAT TCAAATCTTA TAATACCCTT AACTATCATA ACTAA.TATA
Serotype III    TTTTGAATAT GCTACACTAA TTGGTCAGTT TATTTTATTT TCTTATCCCA
Serotype VI     TTTTGAGTAT GCAACACTAT TAGGTCAGTT TATTTTATTC ACTTATCCGA
  Consensus     -****---** -*-*---*-- *---------* ---------*- -**-------

                1051                                                     1100
Serotype IV     TATATGTAAC ACTTTTTTAT AGAAAACTAA GAT.TAATAA CTATTGCTTT
 Serotype V     TGTATATTGT ACTCAATTAT AAACGACTAA AGC.CTGTTG TGATGGTTTT
Serotype Ia     TATATATATA T.TATATGAA GTTAAGAAAC TATTCAATTA TGACCATAGG
Serotype Ib     TATATATATA TATATATTAA GCAAAGATAT AGCTCAGGGA TGATGATACT
Serotype III    TAC.....   TTTTTTTGAA ACCCCAAAAA CATATGGAAA ATATTTTAAT
Serotype VI     TAT.....   TATTTTTAAA ACAGCAGAGG TATGGAGAAA ATATTTTTAT
  Consensus     *--------- --*---*-*- ---------- ---------- --*-------

                1101                                                     1150
Serotype IV     TATTTTAACT CTAAATTACT TCTTGTATCA GTATACTTAT TCAAGAACTG
 Serotype V     ATTTTTAACA TTAAATTATT TATTGTACCA ATATACTTTT TCAAGGACAG
Serotype Ia     TGTTGTATTA TTATTTACCT TTATTTTACC TATTGGATCG GGCTCCAGGG
Serotype Ib     CGGTGCTCTT CTCTCCACTA TTATACTACC CATCGGGTCT GGATCTAGAG
Serotype III    ATCCTTACTG TTGACTATAT GTTCATACTT TTCTGGCGCT AGAATACTAT
Serotype VI     CACACTATTC CTAGTTTTTT GTGCATATTT GACAGGGGCA AGAATTTTCC
  Consensus     ---------- -*-------- ---------- ---------- ----------
```

```
                1151                                                1200
Serotype IV     GATATTATAT AGTACTCTTA TTTATACTTA TTATATATGT TACAAAGAAT
 Serotype V     GGTATTATAT CGTAATTTTA TTTATTGTAC TCATTTATGT GACAAAGAAT
Serotype Ia     CTGGAATAGT AGCTATATTG GCGCAGATGT TTATTCTTCT TCTAAATACA
Serotype Ib     CTGGTATTAT AGTTGTGCTA CTACAGGTTA TAATTTTATT GTTGAATACA
Serotype III    TGGTCTGTAT GTTGGTTTTA TTAGCATCGC TTCTTTTAGA TTATATCCTT
Serotype VI     TAATTTGTAT GATAATTTTA TTAGGTTATT TACTCTTAGA AATAATCATT
  Consensus     ---------* -----*--*- ---------- *--*------ ----*-----

                1201                                                1250
Serotype IV     AACCTGATAA GGAAAATTTT TATGATAGTT GCTCCGTACA TACAACTGTT
 Serotype V     AGCTTAATAA AAAGAGTATT TATGAAATTA GCACCCTATG TACAATTTTT
Serotype Ia     GTTGTCGTAA AGAAGAAAAC TATAAAATTT TTATTGTACA TACTTCCGTT
Serotype Ib     ATTGTAATAA AAAGACAAAC GATAAGATTT TTCCTGTATT TAGTTCCGAT
Serotype III    TTTAAAACTA ATTTGAAATT GACCAAGAAA AACACTTTTA TACTTGGTAT
Serotype VI     AATAAATTTA ACCTAAAAAT TACTAAAAAA GCTGTCTTTT TGATAATTAT
  Consensus     ---------* ---------- -*--*----- ------*--- *--------*

                1251                                                1300
Serotype IV     CTTGTTAGCA TTTACTTTTC TTTGCTCTAC TATTTTTTTC AACTCAAATT
 Serotype V     TTTATTAGTA TTTACCTTTT TGAGTTCTAC AATTTTTTTT AATTCAAATT
Serotype Ia     TCTACTAGTA ATAGTAATGA TGTTATATTT TGATAACTTA CTATCTATAT
Serotype Ib     ACTAATATTA CTATTAGTGA TATTACGTTT TGATAATTTG GTGAGCATAT
Serotype III    GACTTTCTTA TTTATCACCG CTTGTTTTTC TTATAACATA TGGTCAATAA
Serotype VI     AGGGATAATA TTATTATTGG TATGTTTTTC TTACAAAGTG GAGTCTATTA
  Consensus     -----*---* -*-------- --------*-- --------*- ------*---

                1301                                                1350
Serotype IV     TTGTTCAAAA ATTAGATAGC CTTTTGACAG GTAG...... ..........
 Serotype V     TTGTTCAAAA ATTAGATGTT CTTTTAACAG GTAG...... ..........
Serotype Ia     ATTATCGTAT AATTAATTTG CGATCCGGGA GTAGTGAATC CAGATTTTCT
Serotype Ib     ATAATAGAAT AATCAATTTG CGGTCGGGAA GTAGTGAATC TAGATTTTCT
Serotype III    TTGAAAAAAT AATTATGTAC AGAAACCAAA GTACTATCAC TAGGATGATA
Serotype VI     TCAATTATAT AATACACTAT AGATTTCAAA GTAGTAGTAC AAGATTGACA
  Consensus     --------*- *-*------- ---------- ***------- ----------

                1351                                                1400
Serotype IV     ..GTTAAACT ATGCTCATTT ACAGCTTGTA GACGGCTTAA CTCTTTTTGG
 Serotype V     ..ATTACACT ATGCTCATTT ACAACTTGTA GATGGTTTAA CTCCTTTTGG
Serotype Ia     GTATATAAAG ATACAGTAAA CATCGTTATA AATAATTCTT TATTATTTGG
Serotype Ib     TTGTACAAGG ATACCGTACA CTCAGTAATT ACTGACTCAC TATTTCTGGG
Serotype III    GTTTATCAAG AAAGTATTAT TGAAGTTCTA AAAGGAAATA TTTTATTTGG
Serotype VI     GTCTATTACG AAAGTATAAG AGCGATTTTA GATGGGAATT TCCTTATTGG
  Consensus     ---*---*-- *--------- -----*--*- ---------- ------*-**

                1401                                                1450
Serotype IV     AAATAGTTTT AAGGAG.... .......... .........A CGAGTGTCCT
 Serotype V     AAATAGTTTT AAGGAA.... .......... .........A CAAGTGTCCT
Serotype Ia     AGAAGGAGTT AAAGAGTTAT GGTTAAATAG TGATCTACCT TTGGGGTCGC
Serotype Ib     AAAAGGTGTA AAAGAATTGT GGTTAAATAG TGATTTACCA CTAGGATCGC
Serotype III    ACAGGGTATA AGGA...TTC CATCAAGTGA AGGAATATTC CTAGGATCGC
Serotype VI     GCAAGGTATA AGAG...TTC CCTCCAGTGT GGGAATATTT TTAGGTTCAC
  Consensus     --*--*--*- *--------- ---------- ---------- ---*--**--
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                1451                                                1500
Serotype IV     ATTTGATAAT AGCTACTCTA TGTTATTGAG TATGTATGGT GTAGTACTTA
 Serotype V     ATTTGATAAT AGCTACTCTA TGTTATTGAG TATGTATGGT GTAGTACTTA
Serotype Ia     ATTCAACGTA TATAGGCTAT TTCTACAAAA GTGGCCTGCT GGGATTAATG
Serotype Ib     ATTCGACCTA CATAGGTTAT TTCTATAAAA CTGGCCTATT TGGACTAATA
Serotype III    ATTCTACTTA TATTAGTGTC TTTTACAGGA CTTCTTTATT AGGAATTGTT
Serotype VI     ATTCATCATA CATTAGTATA TTTTATAGAA CTTCTTTTAC GGGGCTGTTT
  Consensus     ***------- ---------- *--**----- ------*--- --------*-

                1501                                                1550
Serotype IV     CCATGTTTTG TATGATAATC TATTATATCT ATAGTAAAAA AGTCAATGTA
 Serotype V     CCATGTTTTG TATGATAATC TATTATATCT ATAGTAAAAA GATAATCATA
Serotype Ia     AATATAGTTC CAGGTTTGCT TTTAAT.TTT TACTAATATT GGTAGGAAAG
Serotype Ib     AATGTGATTT TAGGTTTGTT TCTAAT.TCT TATTAGCATT ATCAAGGAAG
Serotype III    CTTTATTTTT CTGCCTTTAT ACTTTTATAT AAAGAAGCGA TTTCAAAAAA
Serotype VI     CTTTTCTTTT CAATATTACT TTTTCTATAT AGAGAAGCTA TCAAACAAAA
  Consensus     -------**- -----*---- --*--*-*-* ---------- ----------

                1551                                                1600
Serotype IV     GTTGAGCTCC AGATACTTTT GTTTA..... .......... ........TA
 Serotype V     ATTGAACTTC AACTACTCCT ATTTA..... .......... ........TA
Serotype Ia     CTAAACAATC AGCTTTTTAT TATGAGATAG TAGGAACACT TATAACTTTA
Serotype Ib     CTAAAAAGTC AGATTTCTAT TATGAGATAG TAGGGTCTGT CATACTCCTA
Serotype III    TTATAAAATC TACAGATTAT TTT....... .........T TTATACGTTA
Serotype VI     CAGGATAATC TACAAGCTTT TTT....... .........T TGGATTGTTA
  Consensus     ----*----* ----------* --*------- ---------- --------**

                1601                                                1650
Serotype IV     ATGTCTATAG TATTATTTAC AGAGAGTTTT TACCCAAGTA TAGTTATGAA
 Serotype V     ATGTCTATAA TATTATTTAC TGAAAGTTTT TATCCCAGTG TGGTAATGAA
Serotype Ia     TTCTCATTTT TTGCACTTGA AGATCTTGAC GGAGCTAATT GGCTTATTGT
Serotype Ib     TTTTCATTTT TTGCACTTGA AGATATTGAT GGCGCCAATT GGCTCATTAT
Serotype III    TTATGTTACA CGCTCTTTGA GGAAATAGAT CCTAATCATT GGAGTATTGT
Serotype VI     TTATTGTATA TGGTATTTGA AGAATTTGAT CCTAATCATT GGAGTGTTGT
  Consensus     -*-*------ ------**-- -**------- --------*- ------*---

                1651                                                1700
Serotype IV     TATTAGTTGG ATGGTTTTTG GGAAAATATT TTGTGGGGGT GTAGATGATT
 Serotype V     TATTAGTTGG CTAGTTTTTG GTAAAATATT TTGTGATGGT ATCGAACCTA
Serotype Ia     TTTTATTTTT ACAGTGTTAG GAATTTTAGA AAATAAGGAT TTTTATAGTC
Serotype Ib     TTTTGTCTTT ACAGTGTTGG GAATTTTAGA AAATAAGGAT TTCTATAGTC
Serotype III    ATTATTATTC TCAACTTTTG GTATAGTGGG AAGGGCTAA. ..........
Serotype VI     ATTGTTATTT ACTACATTAG GTATAGTAGG GAGAG.GGA. ..........
  Consensus     --*----*-- -------**-* *-*---*--- ---------- ----------

                1701                                                1750
Serotype IV     TACAAC.... ...GAGAGTT CACTTGGACG GCAAATAAAA ATTAGTGTAA
 Serotype V     TAAAAA.... ...AGGAATT TACT...ATT GTGAATAATA TATGACATAT
Serotype Ia     AACTTAAAAG GTGGAAAAGT TAATGGAAAA ACGAATACTT GTTTCTATCA
Serotype Ib     AACTTAAAAG GTGGGAAAGT TAATGGAAAA ACAAATACTT GTTTCTATCG
Serotype III    .......... .......... .....AAAAT GAAAGAAAAA GTAACAGTCA
Serotype VI     .......... .......... .....ATGAT AAAAAAACTA GTTAGTGTGA
  Consensus     ---------- ---------- ---------- ---*--*--- -------*--
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                1751                                                    1800
Serotype IV     TTGTACCAGT ATATAATTCG AAACAATATT TAATAGCTTG CGTTGATTCA
 Serotype V     TTGCTCTGAT ATGGCAGGAG GTAAGGAAGG AAAATGATAC CTAAAGTTAT
Serotype Ia     TTATACCTAT ATACAACTCA GAAGCATACC TTAAAGAATG TGTGCAATCC
Serotype Ib     TTATACCTAT ATACAACTCG GAAGCATATC TTAAAGAATG CGTGCAATCC
Serotype III    TTATACCTAT ATACAACTCA GAAGCATACC TTAAAGAATG TGTGCAATCC
Serotype VI     TTGTTCCAGT TTATAATTCG GAGTTAGTGA TTGAGAACTG TGTAGAATCT
  Consensus     **---*---* -*---*---- ---------- ---------- -------*--
                                                    cpsI/M
                1801                                                    1850
Serotype IV     ATTAGAAAAC AAACATATAA GAATTTGGAA ATTATTCTTG TTAATGATGG
 Serotype V     ACATTATTGT TGGTTTGGAG GAAATCCCTT ACCAGATAAT TTAAAGAAAT
Serotype Ia     GTACTACAAC AGACTCATCC ATTGATAGAA GTTATACTAA TTGATGATGG
Serotype Ib     GTCCTACAAC AGACTCATTC ATTGATAGAA GTTATACTGA TTAATGATGG
Serotype III    GTACTACAAC AGACTCATCC ATTGATAGAA GTTATACTAA TTGATGATGG
Serotype VI     TTGCTTCAAC AAACATACCC AGAAATAGAA ATTTTATTAA TAGATGATGG
  Consensus     ---------- ---------- ---------- ---------- *--*-**---

                1851                                                    1900
Serotype IV     ATCAACAGAT GGTAGTAAAG AGTTATGTGA GGAGATAAGA AAATCAGATG
 Serotype V     ATATAAAAA. ...CTTGGAG AGAACAATGT CCGGATTATG AAATTATTGA
Serotype Ia     ATCCACTGAT AATAGTGGAG AAATTTGTGA TAATTTATCT CAAGAAGATA
Serotype Ib     ATCCACTGAT AATAGTGGAG AAATTTGTGA TAATTTATCT CAAAAAGACG
Serotype III    ATCCACTGAT AATAGTGGAG AAATTTGTGA TAATTTATCT CAGGAAGATA
Serotype VI     ATCTACAGAT AAAAGTAGTC ATATTTGTAA TAATTTTTTA AAAAGGGATA
  Consensus     **--*---*- -----*---- *------*-- -----*---- -*--------

                1901                                                    1950
Serotype IV     AAAGAATTAA GACATTTCAC AAAACAAATG GAGGACAATC AAGCGCAAGG
 Serotype V     ATGGAATGAG CATAATTATG ATGTTAGTAA AAATGTTTTT ATGAGAGAAG
Serotype Ia     ATCGCATACT TGTATTTCAT AAAAAAAATG GAGGGGTCTC TTCGGCAAGG
Serotype Ib     ATCGCATACT TGTATTTCAT AAAAAAAATG GAGGGGTATC TTCGGCAAGG
Serotype III    ATCGCATACT TGTATTTCAT AAAAAAAATG GAGGGGTCTC TTCGGCAAGG
Serotype VI     GTCGCGTAAA AGTCTATCAT AAATACAATG GAGGTGCATC ATCAGCAAGA
  Consensus     ---*--*--- ------*--- *---------- -*------*- ----*--*--

                1951                                                    2000
Serotype IV     AATTTAGGTA TTTTATACTC TACAGGAGAT TTGATTGGTT TTGTTGACAG
 Serotype V     CATATACTAA GAAGAATTT. ......TGCT TATGTTTCTG ACTATGCAAG
Serotype Ia     AACCTAGGTC TAGATAAATC CACAGGAGAA TTCATAACAT TTGTGGATAG
Serotype Ib     AACCTAGGTC TTGATAAATC CACAGGCGAA TTCATAACGT TTGTAGATAG
Serotype III    AACCTAGGTC TAGATAAATC CACAGGAGAA TTCATAACAT TTGTGGATAG
Serotype VI     AATGTGGGAC TTGAGATGGC AGAAGGTGAA TTTATAACTT TTGTAGATAG
  Consensus     -*--*----- ---------- -------*-- *---*----- -----*--**

                2001                                                    2050
Serotype IV     CGACGATACA ATTGACCCTA AAATGTATGA AACGTTACTA AATATATATG
 Serotype V     ATTGGATATT ATTTATACTT ATGGGGGGTT CTATCTAGAT ACTGATGTGG
Serotype Ia     TGATGATTTT GTAGCACCGA ATATGATTGA AATAATGTTA AAAAATTTAA
Serotype Ib     TGATGATTTT GTAGCACCGA ATATAATTGA AATAATGTTA AAAAATTTAA
Serotype III    TGATGATTTT GTAGCACCGA ATATGATTGA AATAATGTTA AAAAATTTAA
Serotype VI     CGATGATGTT GTCGCACTAA ATATGATTGA AATTATGCTG AATAATTTGT
  Consensus     ----***--- -*-------- *--------- -----*---- *---------
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                2051                                                2100
Serotype IV     AAGATGAACA AGTAGACTGG GTGCAATGTA ATCACAAAAA AATTTACTCT
 Serotype V     AGCTTTTAAA AAGTTTAGAT CCTTTGAGGA TTCATGAGTG TTTTCTAGCA
Serotype Ia     TCACTGAGAA TGCTGATATA GCAGAAGTAG ATTTTGA... TATTTCGAAT
Serotype Ib     TCACTGAGGA TGCTGATATA GCAGAAGTAG ATTTTGA... TATTTCGAAT
Serotype III    TCACTGAGAA TGCTGATATA GCAGAAGTAG ATTTTGA... TATTTCGAAT
Serotype VI     TAACGGAGAA CGCAGATATA TCAGAAATTG ATTTCGA... AGTTTCAGAT
  Consensus     ---------* ---------- ---------- -*----*--- --**------

                2101                                                2150
Serotype IV     AACGGTGTTA ACTTATATTA TAATGGACCT GAATACTATA ATGTGCTTAA
 Serotype V     AGGGAGATTA GTTGTGATGT GAATACAGGA TTAATAATTG GCGCTGTTAA
Serotype Ia     GAGAGAGATT ATAGAAAGAA GAAAAGACGA AACTTTTATA AAGTCTTTAA
Serotype Ib     GAGAGAGATT ATAGAAAGAA AAAAAGACGA AACTTTTATA AGGTCTTTAA
Serotype III    GAGAGAGATT ATAGAAAGAA GAAAAGACGA AACTTTTATA AAGTTTTTAA
Serotype VI     GA...TTTTT ATAAAAGAAA AAAAAGAAAA GGTTACTATA GAGTTTTTCA
  Consensus     --------*- ---------- -**---*--- --------*- --*---**-*

                2151                                                2200
Serotype IV     TAAACAAGAT TTCCTATACG AATTTCTGAG TACAAATAAG ATTTTTAGTT
 Serotype V     AGGACATCAC TTTTTAAAAT CAAATATGTC TATATATGAC AAAAGTGATT
Serotype Ia     AAACAATAAC TCTTTAAAAG AATTTTTATC AGGCAATAGA GTGGAAAATA
Serotype Ib     AAACAATAAT TCTTTAAAAG AATTTTTATC AGGTAATAGA GTGGAAAATA
Serotype III    GAATAATAAC TCTTTGAAAG AATTTTTATC AGGTAATAGA GTGGAAAATA
Serotype VI     AAACAATAAG TCTCTCAAAG AATTTTTTTC AGGAAATAAA GTAGAAAATG
  Consensus     -----*--*- *---*--*-- -*--*-*--- -----**--- --------*-

                2201                                                2250
Serotype IV     CAGTCTGCGA GGGGTTGTTA TCTAGAGATT TAGCTTTAAA AATAAAATTC
 Serotype V     TAACTTCTCT TAATAAGACA TGTGTAGAGG TTACAACTAA TTTATTGATA
Serotype Ia     TTGTTTGTAC AAAATTATAT AAAAAAAGTA TAATTGGCAA CTTGAGGTTT
Serotype Ib     TTGTTTGTAC AAAATTATAT AAAAAAAGTA TAATTGGTAA CTTGAGGTTT
Serotype III    TTGTTTGTAC AAAATTATAT AAAAAAAGTA TAATTGGTAA CTTGAGGTTT
Serotype VI     TTGTTTGGGG GAAATTATAT AAAAAAAGCA TTATTGGGGA TTTACGATTT
  Consensus     -----*----- ---------- -----*---- *--------* --*-----*-

                2251                                                2300
Serotype IV     CGTGAAGAAA AAAAAT...A TGAAGATACA CAGTTTTATT TTGATCTCAT
 Serotype V     AACAGAGGGC TTAAGA...A TAAGAATATT ATTCAAAAGA TTGA..TGAT
Serotype Ia     GATGAGAACT TAAAAATTGG TGAGGATTTA CTTTTTAATT GTAAACTCTT
Serotype Ib     GATGAGAATT TAAAAATTGG TGAGGATTTA CTTTTTAATT GTAAAATTTT
Serotype III    GATGAGAACT TAAAAATTGG TGAGGATTTA CTTTTTAATT GCAAACTCTT
Serotype VI     AATGAAAAAT ACAAAATTGG TGAAGACTTG CTATTTAACT TTCAGATTTT
  Consensus     ---------- --**------ *-*--*---- -------*-- ---*--*--*

                2301                                                2350
Serotype IV     AAAAAATGCT AATAAGTTTG TTATTATAAG CCAACCTTTT TATAATTACT
 Serotype V     ATAACAATAT ATCCGAGAAA TTATTTTAAT CCAAAGAATT TATTAACA..
Serotype Ia     ATGTCAAGAG CACCGTATAG TCGTAGATAC GACTTCTTCC TTATATACTT
Serotype Ib     ATGTCAAGAG CACTGCATAG TCGTAGATAC GACTTCTTCC TTGTACACCT
Serotype III    ATGTCAAGAG CACCGTATAG TCGTAGATAC GACTTCTTCC TTATATACTT
Serotype VI     AAATAAAGAA CATCGTATAG TTGTAGATAC TAGAAGATCA CTCTATACTT
  Consensus     *----*---- ---------- *--*----*- ---------- ----*-----
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                2351                                                2400
Serotype IV     ACTACAGAAA AAATAGTACA ACAACTTCCT CATATAGTAG CTATCAATGG
 Serotype V     ..GGTAAGGT TGATTGTCTG ACTAGTGTTA CCTATTCTAT ACATCATTAC
Serotype Ia     ATCGAATTGT AAAAACTTCC GCAA.TGAAT CAGAAATTCA ACGAAAACTC
Serotype Ib     ATCGCATCGT AAAGACTTCT GCAA.TGAAT CAGGAGTTCA ACGAAAATTC
Serotype III    ATCGAATTGT AAAAACTTCT GTAA.TGAAT CAGAAATTCA ACGAAAACTC
Serotype VI     ATCGTATTGA AGAAAAATCT ATAA.TGAAT CAACAATTTA ATAAAAATAC
  Consensus     -----*---- --*------- ---*-*---- *-------*-- -----*----

                2401                                                2450
Serotype IV     GACATAATCG ATATCTGTAC TGAGTGTTAT TATTATGCAA AGGATTTTAA
 Serotype V     GAAGGAAGTT GGAAAAGTTC TTCATTTATT TCAGATTCTC TAAAGATTAG
Serotype Ia     ATTAGATTTT ATAACAATTT TTAATGAAGT AAGTAGTTTG GTTCCTGCCA
Serotype Ib     ATTAGATTTT ATAACAATTT TTAATGAAAT AAGCAGTATT GTTCCTGCAA
Serotype III    ATTAGATTTT ATAACAATTT TTAATGAAAT AAGTAGTTTG GTTCCTGCCA
Serotype VI     ATTAGACTTC ATTGATATTT TTAATGAGAT TCATCAGGAT AGTCCGACAG
  Consensus     -----*---- -------*-- *---*----* ---------- ----------

                2451                                                2500
Serotype IV     TGGATTTGAA GAAGTTGCTT TTTCAAGATT ATTTGGTGCA TATTCGTTAG
 Serotype V     AGTAAGGCTC ATAATTGATT TTTTATTTGG ATATGGTACT TATAGAATGC
Serotype Ia     AATTGGCTAA TTATGTTGAA GCGAAATTTT TAAGAGAAAA GATAAAGTGT
Serotype Ib     AATTAGCTAA TTATGTTGAA GCGAAATTTT TAAGAGAAAA GGTAAAGTGT
Serotype III    GATTAGCTAA TTATGTTGAA GCGAAATTTT TAAGAGAAAA GATAAAGTGT
Serotype VI     AATTGTTTAA TTATGTGGAA GCGAAGTTTG TACGAGAAAA AATCAAGTGT
  Consensus     ---------- --*--*---- ----*----- -----*---- --*----*--

                2501                                                2550
Serotype IV     TAGCTAATAA AATTGTATAT AATAAAGATT ATAGAAAAAC CGAAGAATTA
 Serotype V     TTCTAAGGTT TCTAAAGTTA AAGAAATAG. .......... ..........
Serotype Ia     CTCCGAAAAA TGTTTGAATT AGGTAGTAAT ATTGACAATA AAATCAAAGT
Serotype Ib     CTCCGAAAAA TGTTTGAATT AGGTAGTAAT ATTGACAGTA AAATCAAATT
Serotype III    CTCCGAAAAA TGTTTGAATT AGGTAGTAAT ATTGACAATA AAATCAAAGT
Serotype VI     TTAAGGAAAA TGTTTGAATT AGGAGAAATA GCTGATGAAA ATTTACGTTT
  Consensus     ---------- --*------- *------*-- ---------- ----------

                2551                                                2600
Serotype IV     AGATAA.... .......... .......... .......... ..........
 Serotype V     .......... .......... .......... .......... ..........
Serotype Ia     ACAACGAGAG ATTTTTTTCA AAGACATTAA ATCATACCCG TTCTATAAAG
Serotype Ib     ACAACGAGAG ATTTTTTTCA AAGATGTTAA ATTATACCCT TTCTATAAAG
Serotype III    ACAACGAGAG ATTTTTTTCA AAGACATTAA ATCATACCCG TTCTATAAAG
Serotype VI     ACAGAGATAT AAATTTTGGC AAGATATTAA ATCATATTCA ATATGCAAAG
  Consensus     ---------- ---------- ---------- ---------- ----------

                2601                                                2650
Serotype IV     .......... .......... .......... .......... ..........
 Serotype V     .......... .......... .......... .......... ..........
Serotype Ia     CGGTAAAATA CTTATCATTA AAGGGATTAT TAAGCTTTTA TTTAATGAAA
Serotype Ib     CGGTTAAGTA CTTATCATTA AAGGGATTAT TGAGTATTTA CTTAATGAAA
Serotype III    CGGTCAAATA CTTATCATTA AAGGGATTAT TAAGCTTTTA TTTAATGAAA
Serotype VI     CAATAAGGTT CTTATCTAAA AAACATATCT GTACGTTATA TTTGATGAAA
  Consensus     ---------- ---------- ---------- ---------- ----------
```

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                2651                                                  2700
Serotype IV     .......... .......... .......... .......... ..........
 Serotype V     .......... .......... .......... .......... ..........
Serotype Ia     TGTTCACCTA AACTATATGT TATGGCATAT AGAAGATTCA AAACAGTAGC
Serotype Ib     TGTTCACCCA TCTTGTATAT AAAATTATAT GACAGGTTTC AAAAACAGTA
Serotype III    TGTTCACCTA AACTATATGT TATGGCATAT AGAAGATTTC AAAAACAGTA
Serotype VI     TATTTTCCGT ACGTATATAT AAAGATGTAT AATAAATTTC AAAAGCAATA
  Consensus     ---------- ---------- ---------- ---------- ----------

                2701                  2728
Serotype IV     .......... .......... ........
 Serotype V     .......... .......... ........
Serotype Ia     TGGAGAAATT GGGAAAGAGA ATTTATAA
Serotype Ib     A......... .......... ........
Serotype III    G......... .......... ........
Serotype VI     A......... .......... ........
  Consensus     ---------- ---------- --------
```

Notes.
Serotype Ia: GenBank accession number AB028896;
Serotype Ib: GenBank accession number AB050723;
Serotype III: GenBank accession number AF163833;
Serotype IV: GenBank accession number AF355776;
Serotype V: GenBank accession number AF349539;
Serotype VI: GenBank accession number AF337958.

Figure 4. Two sites (*) of sequence heterogeneity between *alp2* (AF208158, upper lines) and *alp3* (AF291065, lower lines) used to distinguish them (relevant primers are shown).

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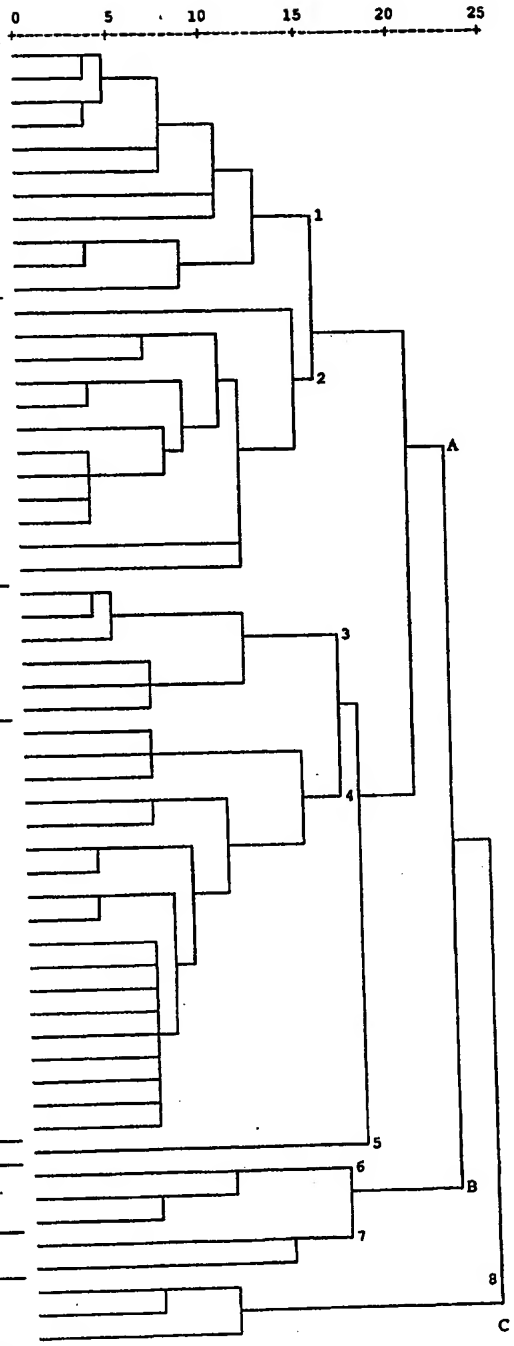
251 AAGGTAATCTTAATATTTTTGAAGAGTCAATAGTTGCTGCATCTACAATT 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
531 AAGGTAATCTTAATATTTTTGAAGAGTCAATAGTTGCTGCATCTACAATT 580
              .                bcaS1 .       .       .
301 CCAGGGAGTGCAGCGACCTTAAATACAAGCATCACTAAAAATATACAAAA 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
581 CCAGGGAGTGCAGCGACCTTAAATACAAGCATCACTAAAAATATACAAAA 630
              .     bcaS2     .       .       .
351 CGGAAACGCTTACATAGATTTATATGATGTAAAGAATGGATTGATTGATC 400
    ||||||*|||||||||||||||||||||||||||||||||||||*||||
631 CGGAAATGCTTACATAGATTTATATGATGTAAAGAATGGATTGATCGATC 680
              .       .       .       .       .
401 CTCAAAACCTCATTGTATTAAATCCATCAAGCTATTCAGCAAATTATTAT 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
681 CTCAAAACCTCATTGTATTAAATCCATCAAGCTATTCAGCAAATTATTAT 730
              .       balS    .       .       .
451 ATCAAACAAGGTGCTAAATATTATAGTAATCCGAGTGAAATTACAACAAC 500
    ||||||||||||||||||||||||||||||||||||||||||||||||||
731 ATCAAACAAGGTGCTAAATATTATAGTAATCCGAGTGAAATTACAACAAC 780
              .       .       .       .       .
501 TGGTTCAGCAACTATTACTTTTAATATACTTGATGAAACTGGAAATCCAC 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||
781 TGGTTCAGCAACTATTACTTTTAATATACTTGATGAAACTGGAAATCCAC 830
              .       .       .       .       .
551 ATAAAAAAGCTGATGGACAAATTGATATAGTTAGTGTGAATTTAACTATA 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||
831 ATAAAAAAGCTGATGGACAAATTGATATAGTTAGTGTGAATTTAACTATA 880
              .       .       .       .       .
601 TATGATTCTACAGCTTTAAGAAATAGGATAGATGAAGTAATAAATAATGC 650
    ||||||||||||||||||||||||||||||||||||||||||||||||||
881 TATGATTCTACAGCTTTAAGAAATAGGATAGATGAAGTAATAAATAATGC 930
              .       .       .       .       .
651 AAATGATCCTAAGTGGAGTGATGGGAGTCGTGATGAAGTCTTAACTGGAT 700
    ||||||||||||||||||||||||||||||||||||||||||||||||||
931 AAATGATCCTAAGTGGAGTGATGGGAGTCGTGATGAAGTCTTAACTGGAT 980
                              balA
```

Figure 5

Genetic markers of GBS genotypes	Strain No. (%)	Genotypes
V-alp3-IS*1381*-ISSa*4*	2 (1)	V-2
V-alp3-IS*1381*-IS*861*-ISSa*4*	1 (0.5)	V-3
V-alp3-IS*1381*	27 +1 (14)	V-1
V-alp3	1 (0.5)	V-7
V-alp3-IS*1381*-IS*861*-GBSil	1 (0.5)	V-4
Ib-alp3-IS*1381*	1 (0.5)	Ib-14
V-None-GBSil	1 (0.5)	V-6
III-alp3-IS*1381*-GBSil	1 (0.5)	III-4-2
II-alp3-IS*1381*-IS*1548*-GBSil	1 (0.5)	II-10
II-alp3-IS*1381*-IS*1548*	1 (0.5)	II-8
II-alp3-IS*1381*-IS*861*	1 (0.5)	II-9
II-a-IS*1381*-ISSa*4*-GBSil	2 (1)	II-4
VI-as-IS*1381*	1 (0.5)	VI-4
VI-a-IS*1381*	1 (0.5)	VI-5
IV-a-IS*1381*	5 (3)	IV-1
IV-a-IS*1381*-IS*861*	1 (0.5)	IV-2
Ia-as-IS*1381*	3 (2)	Ia-2
Ia-a-IS*1381*-IS*861*	3 (2)	Ia-3
Ia-a-IS*1381*	34+1 (18)	Ia-1
Ia-None-IS*1381*	1 (0.5)	Ia-8
Ia-a-IS*1381*-ISSa*4*	1 (0.5)	Ia-4
Ia-AaB5a-IS*1381*-IS*861*	1 (0.5)	Ia-6
Ia-a-IS*861*-GBSil	1 (0.5)	Ia-5
II-Aa-IS*1381*-IS*861*-ISSa*4*	2 (1)	II-2
II-AaB9-IS*1381*-IS*861*-ISSa*4*	1 (0.5)	II-7
II-AaB4-IS*1381*-IS*861*-ISSa*4*	1 (0.5)	II-5
II-AaB1-IS*1381*-IS*861*-IS*1548*	2 (1)	II-3
II-Aa-IS*1381*-IS*861*-IS*1548*-GBSil	1+1 (1)	II-12
II-AaB4-IS*1381*-IS*861*-IS*1548*	1 (0.5)	II-6
VI-AaB3b-IS*1381*-IS*861*	+1 (0.5)	VI-2
VI-Aa-IS*1381*-IS*861*-GBSil	1 (0.5)	VI-3
VI-AaB7a-IS*1381*-IS*861*	1 (0.5)	VI-1
Ib-AaB3c-IS*1381*	1 (0.5)	Ib-6
Ib-AaB7-IS*1381*	1 (0.5)	Ib-9
Ib-AaB10-IS*1381*-IS*861*	1+1 (1)	Ib-13
Ib-AaB10-IS*1381*-IS*861*-ISSa*4*	1 (0.5)	Ib-12
Ib-AaB1-IS*1381*-IS*861*	13+2 (8)	Ib-1
Ib-AaB1-IS*1381*	1 (0.5)	Ib-2
Ib-AaB3-IS*1381*-IS*861*	2 (1)	Ib-5
Ib-AaB4b-IS*1381*-IS*861*	1+1 (1)	Ib-8
Ib-AaB8-IS*1381*-IS*861*	1 (0.5)	Ib-10
Ib-AaB4-IS*1381*-IS*861*	1 (0.5)	Ib-7
Ib-AaB1a-IS*1381*-IS*861*	1 (0.5)	Ib-3
Ib-AaB2-IS*1381*-IS*861*	1 (0.5)	Ib-4
Ib-AaBN1-IS*1381*-IS*861*	1 (0.5)	Ib-15
Ib-AaBN2-IS*1381*-IS*861*	1 (0.5)	Ib-16
Ib-AaB9a-IS*1381*-IS*861*	1 (0.5)	Ib-11
III-4-AaB2-IS*1381*-IS*861*-GBSil	1 (0.5)	III-4-1
III-R-IS*861*-GBSil	13+4 (9)	III-2
II-R-IS*1381*-IS*861*-GBSil	4 (2)	II-1
IV-R-IS*1381*-IS*861*-GBSil	1 (0.5)	IV-3
III-R-IS*1381*-IS*861*-IS*1548*	22+4 (13)	III-1
V-RB3a-IS*1381*-IS*861*	1 (0.5)	V-5
III-alp2as	3+1 (2)	III-3
Ia-alp2as	1 (0.5)	Ia-7
II-alp2as-GBSil	1 (0.5)	II-11
Total=56 genotypes	Total=177+17	56 genotypes



INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01281

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12Q 1/68, 1:46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS: CA: MEDLINE. KEYWORDS: Streptococcus, Streptococcus agalactiae, cps, capsular polysaccharide, capsular antigen, sero?, rib, alp2, alp3, surface antigen, surface protein, mobile genetic element, transposon.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Kong et al. "Serotype Identification of Group B Streptococci by PCR and Sequencing" Journal of Clinical Microbiology (2002) Vol 40 (1): pages 216-226 (see the whole document)	1-12, 23-28 31-35
P, X	Kong et al. "Molecular Profiles of Group B Streptococcal Surface protein Antigen Genes: Relationship to Molecular Serotypes" Journal of Clinical Microbiology (2002) Vol 40 (2): pages 620-626 (see the whole document)	13-17, 29-35
X	Lachenauer et al. "Mosaicism in the Alpha-Like Protein Genes of Group B Streptoccoci" Proc Natl Acad Sci USA. (2000) Vol 97 (17): pages 9630-9635 (see the whole document) EMBL DATABASE AF208158, ACCESSION NUMBER AF208158 23 August 2000	13-16, 29

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
28 November 2002

Date of mailing of the international search report
09 DEC 2002

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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01281

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Tamura et al. "Analysis of Restriction Fragment Length Polymorphisms of the Insertion Sequence IS1381 in Group B Streptococci" The Journal of Infectious Diseases (2000) Vol 181: pages 364-368 (see the whole document)	18-20
X	Yamamoto S et al. "Molecular characterization of type-specific capsular polysaccharide biosynthesis genes of Streptococcus agalactiae type Ia Journal of Bacteriology (1999) Vol 181: pages: 5176-5184 EMBL DATABASE ENTRY AB028896.2, ACCESSION NUMBER AB028896 16 July 1999	23
X	Miyake K et al. "CpsJ of Streptococcus agalactiae type Ib shows beta-1,3-galactosyltransferase activity" Unpublished. EMBL DATABASE ENTRY AB050723.1, ACCESSION NUMBER AB050723 5 February 2001	27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01281

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Invention I: Claims 1 to 12, 23 to 28 completely and claims 17, 22, and 31 to 35 partially.

Invention II: Claims 13 to 16, 29 and 30 completely and claims 17, 22, and 31 to 35 partially.

Invention III: Claims 18 to 21 completely and claim 22 partially. (see supplemental sheet)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01281

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: II

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. In coming to this conclusion the International Searching Authority has found that there are different inventions as follows:

Invention I relates to primers, compositions and methods for typing group B streptococcus based on sequence analysis of capsular proteins *cps*D, *cps*E, *cps*F, *cps*G and/or *cps*I/M.

Invention II relates to primers, compositions and methods for typing group B streptococcus based on determining the presence or absence of one or more of the surface protein genes, *rib, alp2* and *alp3*.

Invention III relates to primers and methods for typing group B streptococcus based on determining the presence or absence of one or more mobile genetic elements selected from IS*861*, IS*1548*, IS*1381*, ISSa*4* and GBSi1.

The technical feature common to all three inventions is considered to be methods of typing group B streptococcus using nucleic acid primers. However this feature is obvious in view of the disclosure of Lachenauer et al. which prefigures methods of typing based on mosaicism in the *alp*2 and *alp*3 genes in GBS strains V and VIII. Therefore methods of typing group B streptococcus can not be regarded as a unifying technical feature.